Changes In Leukocyte Subsets And Anti-dsDNA Antibody Levels After B cell Depletion Therapy In Systemic Lupus Erythematosus

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Declaration

I, Mark Lazarus, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Clinical response to B cell depletion therapy (BCDT) is highly variable in patients with systemic lupus erythematosus (SLE). Reductions in anti-dsDNA antibody levels also vary. It has been shown that early relapse is more likely if anti-dsDNA antibody levels remain high after BCDT. The cellular factors that determine how anti-dsDNA antibodies are produced and whether they are likely to fall after BCDT have not been clarified. This thesis describes two different immunological processes that might explain why anti-dsDNA antibody levels either fall or remain high after BCDT. (1) In patients whose anti-dsDNA antibody levels fall, it was hypothesized that short-lived plasma cells arise from ectopic lymphoid tissue (ELT). They had higher percentages of $CD4^+$ T cells expressing chemokine receptors CXCR3, CCR5 and the integrin CD49d, which are associated with migration into non-lymphoid tissue. Clinical relapse in this group starts with the expansion of B cells in the blood, in particular the IgD^- $CD27^-$ subset, and is followed by a fall in circulating lymphocyte numbers before anti-dsDNA antibody levels rise. $CD4^+$ T cell numbers are inversely related to HLA-DR expression by $CD4^+$ T cells and $CD4^+CD49d^{hi}$ T cells express HLA-DR, suggesting that HLA-DR induces T cell migration. Activated B cells can induce HLA-DR expression by T cells, suggesting that they initiate T cell migration. Taken together, the data suggest that B cells induce the formation of ELT. (2) Patients with anti-dsDNA antibody levels that do not fall after BCDT had higher percentages of $CD4^+$ T cells that express CCR7 and CD62L, which are associated with migration to secondary lymphoid organs (SLO), supporting data that show long-lived plasma cells are produced in SLOs. In conclusion, T cell surface markers might be useful for predicting the clinical outcomes and serological changes following BCDT and designing therapeutic trials in SLE.

Publications and Posters

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Posters:

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Abbreviations

ACR	- American College of Rheumatology
APC	- antigen presenting cell/ allaphocyanin
ANA	- antinuclear antibody
APRIL	- a proliferation-inducing ligand
ASC	- antibody-secreting cell
BCDT	- B cell depletion therapy
bd	- twice per day
BAFF	- B cell activating factor
BAFF-R	- B cell activating factor receptor
BCMA	- B cell maturation antigen
BCR	- B cell receptor
BILAG	- British Isles Lupus Assessment Group
BLyS	- B Lymphocyte Stimulator
C	- complement
CCR	- chemokine receptor
CD	- cluster of differentiation
CLP	- common lymphoid progenitor
CTLA	- cytotoxic T-lymphocyte antigen
CXCL	- chemokine ligand
CXCR	- chemokine receptor
CpG	- cytosine-phosphate-guanine
DC	- dendritic cell
DMSO	- dimethyl sulphoxide
DNA	- deoxyribonucleic acid
ECLAM	- European Consensus Lupus Activity Measurements
EDTA	- ethylenediaminetetraacetic acid
ELT	- ectopic lymphoid tissue
ENA	- extractable nuclear antigen
Fc	- fragment, crystallizable
FCS	- foetal calf serum
FDC	- follicular dendritic cell
FITC	- fluorescein isothiocyanate
FoxP3	- forkhead box P3
GITR	- glucocorticoid-induced TNFR family related gene
HCG	- human chorionic gonadotrophin
HLA-DR	- human leukocyte antigen, DR subtype
ICAM	- intercellular adhesion molecule
ICOS	- inducible T-cell costimulator
IFN	- interferon
Ig	- immunoglobulin
IL	- interleukin
ILE	- incomplete lupus erythematosus
IP	- interferon gamma-induced protein
LFA	- lymphocyte function-associated antigen
LLPC	- long-lived plasma cell
MDC	- myeloid dendritic cell
MCP	- monocyte chemoattractant protein
MFG-E8	- milk fact globule-EGF factor 8 protein
MHC	- major histocompatibility complex
MZ	- marginal zone
NK	- natural killer
NKT	- natural killer T

NLR	- NOD-like receptor
od	- once per day
PAMP	- pathogen associated molecular pattern
PBMC	- peripheral blood mononuclear cell
PBS	- phosphate buffer solution
PDC	- plasmacytoid dendritic cell
PE	- phycoerythrin
PE-Cy	- phycoerythrin-cyanine
PRR	- pathogen recognizing receptor
RANTES	- regulated on activation, normal T cell expressed and secreted
RCT	- randomized controlled trial
RNA	- ribonucleic acid
RPMI	- Roswell Park Memorial Institute medium
SCID	- severe combined immunodeficiency
SDF	- stromal cell derived factor
SLAM	- systemic lupus activity measure
SLE	- systemic lupus erythematosus
SLEDAI	- systemic lupus erythematosus disease activity index
SLICC	- Systemic Lupus International Collaborating Clinics
SLO	- secondary lymphoid organ
SLPC	- short-lived plasma cells
TACI	- transmembrane activator and calcium modulator and cyclophilin ligand interactor
TARC	- thymus and activation-regulated chemokine
TCR	- T cell receptor
tds	- three times per day
TGF	- transforming growth factor
TNF	- tumour necrosis factor
TLR	- toll-like receptor
VCAM	- vascular cell adhesion molecule
VLA	- very late antigen

“The greatest thing a human soul ever does in the world is to see something and tell what it saw in a plain way. Hundreds of people can talk for one who can think, but thousands can think for one who can see. To see clearly is poetry, prophecy and religion all in one.”

John Ruskin

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1 INTRODUCTION

1.1.1 The immune system

All living organisms have evolved an immune system, which has mechanisms that can detect, neutralize and clear unwanted substances to protect them from the environment. The immune system is broadly divided into the innate immune system, which can be found in all organisms including single cell organisms, such as protozoa, and the adaptive immune system, which is found only in vertebrates (1).

The central function of the immune system is to prevent infection or invasion, whether by other living organisms, such as bacteria, fungi and helminths, by non-living replicating agents, such as viruses, or by proteins, such as clostridial toxins (2, 3). A number of mechanisms are required, including creating barriers to prevent invasion and clearance (4). However, in higher vertebrates the immune system has at least 3 additional functions. One of these functions is to clear the body of unwanted by-products of cell metabolism, such as oxidized lipoproteins (5). A second is to clear the body of by-products of cell death, which are either released spontaneously, due to necrosis, or by an organized process called apoptosis (6-8). A third important function is the control of excessive cell growth and division (malignancy) (9).

In vertebrates innate immunity provides the initial immune response followed by the adaptive immune response. These responses are mediated by white blood cells, called leukocytes. Leukocytes respond to molecules, which are termed antigens. They can originate from the host (self or auto-antigen) or from the external environment (foreign or non-self antigen). These antigens can be in various different forms of protein, lipid, carbohydrate or nucleic acid. Leukocytes also vary in their ability to detect these different forms (1).

1.1.2 Anatomy of the immune system

The immune system of vertebrate animals is composed of multiple different structures, of which five interface with the environment; the skin, gastrointestinal tract, genitourinary tract,

eyes and lungs. Each of these interfaces are covered by a layer of epithelial cells which differentiate to allow (or actively transport) entry of substances that are necessary for the body but prevent and contain invasion by microorganisms, sometimes by secreting antimicrobial peptides (10, 11).

White blood cells (or leukocytes) that circulate within the body are made in the bone marrow and then, in the case of a specialized group of leukocytes called T cells, undergo further differentiation and selection in the thymus. These 2 anatomical structures are referred to as the primary lymphoid organs. From these organs the leukocytes then enter the blood and lymphatic vessels where they subsequently migrate to the spleen or lymph nodes, which are referred to as the secondary lymphoid organs (SLO) (12). The liver also forms a critical part of the immune system both by producing important proteins and lipids (13) and providing a place for leukocytes to survey the blood for pathogens (14).

The leukocyte populations and immune responses differ in the non-lymphoid tissue depending on the tissue function and its environment. The gastrointestinal tract, for example, has highly specialized lymphoid tissue to manage the higher exposure of the cells to microorganisms and ingested food (15). The brain on the other hand has no lymphoid tissue and limits the infiltration of leukocytes from the circulation in order to prevent excessive immune responses within it. It instead relies mostly on a number of brain-specific innate immune cells to protect it from infection and clear products of tissue damage (16).

1.1.3 The innate immune system

Innate immunity provides the first line of defense against microbial invasion and detection of cellular damage or dysfunction (stress). The response is generally rapid but of limited specificity and self-regulation, being mainly dependent on clearance of the stimulating proteins or organisms before the response subsides. The innate immune system is composed of epithelial barriers, soluble proteins, circulating effector cells and cytokines. Stimulation and inhibition of the innate immune system is dependent on a limited number of pattern

recognition receptors (PRR) that are encoded in the germline deoxyribonucleic acid (DNA) and detect structures that are commonly seen on a wide variety of microbes but not in the host. These receptors include toll-like receptors (TLR), Nod-like receptors (NLR), C-type lectins and scavenger receptors (17, 18).

The cells of the innate immune system include mononcuclear phagocytes, such as macrophages and dendritic cells (DC), polymorphonuclear phagocytes, such as neutrophils, and natural killer (NK) cells. Their primary function is to clear the tissue of the invading pathogen by killing it or the cell it has invaded. However, the cells also secrete messenger molecules, called cytokines, to recruit other cells to the infected tissue or activate cells, such as B cells. Macrophages and DCs can also process the antigen that it has captured from the tissue and present it in the form of peptide with the major histocompatibility complex (MHC) on its cell surface to T cells. If these antigen-presenting cells (APCs) have received an additional signal via one of its PRRs then it will be activated to provide additional stimulation to the T cell, by co-stimulation and/or cytokine secretion, resulting in the activation and differentiation of the T cell. Therefore, innate cells and their PRRs also have an important role in directing the adaptive immune response. However, both B and T also have functional PRRs that can directly influence their differentiation, which shows that there is not a clear boundary between innate and adaptive immunity (19-21).

1.1.4 The adaptive immune system

The evolutionary origins of the adaptive immune system can be traced to the vertebrates (22). A number of potential environmental pressures may have contributed to its development but it is probably not a coincidence that animals with adaptive immunity are significantly bigger than those without and are characterized by more compartmentalized internal organs, in particular the brain. Adaptive immunity supports these physical differences by having mechanisms that are able to react to a wide range of pathogens, localize to an infected compartment, expand and then contract when the threat is cleared. It can then form a memory

for the pathogen to allow it to respond more rapidly if reinfected whilst occupying only a small area within the body and using a minimal amount of energy (1).

The formation of memory cells following initial exposure to an antigen is called the primary immune response. The process starts with antigen recognition, followed by clonal expansion and differentiation. Once the antigen is cleared most of the cells die by apoptosis, a process termed contraction, but a few survive to become memory cells. With re-stimulation, a secondary immune response can expand more rapidly and migrate to the invaded or stressed tissue. It contracts again via cell apoptosis once the pathogen has been removed (23-26).

There are two different types of adaptive immune response, humoral and cell-mediated, which are both controlled by a specialized group of cells called lymphocytes. Humoral immunity is mediated by small molecules called antibodies that are secreted into the blood or mucosa by B lymphocytes (or B cells). Cell mediated immunity involves the recruitment of a variety of different leukocytes, depending on the pathogen, under the control of T lymphocytes (T cells). Each B and T cell in the body has a receptor that is specific to a particular antigen. However, these cells might share the same receptor if they arise from the same clone (27).

The immune response that results from a stimulus is highly specific to the invading microorganism. In order to be able to respond to the multiple different types of microorganisms the adaptive immune system requires very high receptor diversity, which is achieved via gene rearrangement. However, the process can produce receptors that recognize self-antigens. To prevent inappropriate immune responses against the cells of the host lymphocytes that recognize self-antigen are either removed, made unresponsive (anergic) or induced to become regulatory cells. This process occurs either in the bone marrow or the thymus (central tolerance) or in the non-lymphoid tissue (peripheral tolerance) (27-29).

1.1.5 Lymphocytes

Mediated by the cytokine interleukin 7 (IL-7), which is produced locally by stromal cells in the bone marrow, B cells, T cells and NK cells originate from a common lymphoid progenitor cell (CLP) (30). Unlike B and T cells, NK cells have only a limited range of receptors and therefore are considered part of the innate immune system. However, B and T cells differ in the nature of their receptors and their specific function, which is discussed below.

B cells

There are three important subsets of mature B cells; follicular B cells, marginal zone (MZ) B cells and B-1 B cells. The primary and unique function of B cells is to produce antibodies by differentiating into antibody secreting plasma cells, but plasma cells can be short or long-lived, migrate to different locations and the antibodies that they produce can vary in isotype and function. B cells also have additional functions, such as antigen-presentation and cytokine production, which can vary between cells (31). However, the precise factors that determine their differentiation and activation, particularly in humans, remain unclear.

Both MZ and follicular B cells arise from the CLP, which commits itself to the B cell lineage following expression of specific transcription factors, in particular Pax-5, that regulate the expression of genes specifically involved in B cell signaling and gene recombination (30). With the expression of these genes B cells go through a number of stages of maturation from pro-B cell, to pre-B cell, to immature B cell and then to the mature B cell. Each stage is characterized by the expression of a specific combination of surface markers (Table 1.1.1) and immunoglobulin (Ig) genes. The Ig gene rearrangement eventually forms an intact IgM molecule (the B cell receptor (BCR)) on the surface of the immature B cell (32, 33). The rearrangement is an important process that contributes to the BCR diversity. During this stage B cells will be exposed to a number of antigens, primarily in the bone marrow. If the BCR binds to an antigen with high affinity it will be induced to edit the BCR or eliminated. If no antigen is detected the B cell survives. Immature B cells leave the bone marrow and then enter the peripheral lymphoid tissue where they undergo further selection and maturation,

controlled by B cell activating factor (BAFF), by first differentiating into transitional B cells and then either follicular B cells, which recirculate through the blood and follicles in the SLOs and bone marrow or marginal zone B cells, which reside mostly near the marginal sinus of the spleen (i.e. outside the follicle) (31, 34).

Follicular B cells go through several further rounds of selection and activation, but this time with the help of T cells, in particular the T follicular helper (T_{FH}) cell. These T cells interact with mature B cells at the B:T interface of the follicular niche in the SLO where B cells will present antigen to the T cell (35). Using a combination of signals, including cytokines, such as IL-4 and IL-21, and co-stimulatory molecules, such as CD40 ligand, T_{FH} cells instruct the B cells to differentiate into either short-lived extrafollicular plasma cells, which appear to be important for the primary immune response (36), early memory B cells or germinal centre (GC) B cells. To enter the centre of the follicle GC B cells downregulate the chemokine receptor EBV-induced molecule 2 (EBI2) where they encounter antigen-bearing follicular DCs (FDC) and more T_{FH} cells (37). GC B cells undergo somatic hypermutation, which changes the affinity of the BCR. Those cells with the BCR of highest affinity will out compete GC B cells with BCRs of lower affinity for antigen from the FDC and receive the most survival signals from the surrounding T_{FH} cells after presenting antigen to them. Therefore, the GC B cells with high affinity BCRs are more likely to clonally expand and differentiate into plasmablasts. T_{FH} cells provide additional co-stimulatory and cytokine signals to induce GC B cells to either become long-lived memory B cells, long-lived plasma cells or re-enter the cell cycle.

Marginal zone and B-1 B cells can produce some antibodies without conventional help from T cells, i.e. via T cell receptor (TCR) and MHC class II interaction. The antigens that these antibodies bind to are not proteins and are often multivalent with repeating determinants, such as polysaccharides and nucleic acids. They are called thymus independent (TI) antigens. There are three types of TI-antigens. Each of these antigens requires help from a different signal to induce an antibody response; TI-1 antigens, which are typically lipopeptides,

lipopolysaccharides or nucleic acids, require TLR stimulation, TI-2 antigens, which are polysaccharides, require crosslinking of several BCRs leading to a prolonged signal to the B cell, and TI-3 antigens, which are microbial genetic material, require myeloid cells to provide additional signals, such as BAFF and IL-21. T cell-derived CD40L and IL-21 can enhance the TI-1 antibody response. The humoral response can be rapid, suggesting that they have an important role in the initial immune response. However, memory B cells and long-lived plasma cells can be formed, even though they do not arise from the germinal centres in SLOs (38).

The secreted antibody is formed by means of class-switch recombination (CSR), which involves switching the component of the receptor attached to the cell membrane, known as the heavy chain, into one of several secretory forms. Change to the heavy chain is known as isotype switching. Each antibody isotype differs in their ability to bind to Fc receptors, which are expressed on leukocytes, and induce complement activation. They also differ for half-life and tissue distribution. Therefore, they are utilized for different immune responses. IgE, for example, elicits a mast cell response that is good at clearing helminths (39, 40), whilst IgG forms immune complexes that activate complement, which opsonize bacteria (41). CSR can occur at various stages after B cell maturation. T cells can induce CSR, using cytokines and co-stimulation, but are not always necessary, because other cells, such as DCs and even TLR-stimulated epithelial cells can also provide the necessary signals (42, 43).

	Pro-B	Pre-B	Immature	Transitional	Mature Naïve	Mature Pre-GC	GC	Memory	Plasma Cell
CD19	+	+	+	+	+	+	+	+	low
CD20	–	+	+	+	+	+	+	+	–
CD38	high	high	high	high	–/low	high	high	–/low	high
CD10	high	high	int	–	–	+	+		
CD24	high	high	high	high	int				
IgM			+	high	+	+			
IgD				–/low	+	+	–	–	–
CD27							+	+	+

Table 1.1.1 – Surface markers of human B cell subsets
(33). GC; Germinal centre.

Antibody-independent roles of B cells

Although the primary function of B cells is to differentiate into antibody secreting plasma cells, it has also been known that they can present antigen, express co-stimulatory molecules and secrete cytokines. Studies have shown that these different functions can prime $CD4^+$ T cells (44, 45), generate $CD4^+$ memory T cells (46) and they can directly influence the differentiation and polarization of T cells (47). They might also play a role in preventing or suppressing an immune response by inducing a population of regulatory T cells (47). They have also been shown to express a range of different chemokines, suggesting that they can recruit other immune cells to SLOs or non-lymphoid tissue (48-50).

It is not clear which B cell subsets are involved in these non-antibody producing roles and what stage of the immune response they are important for mediating. B1 and MZ B cells produce more IL-10, which induces regulatory T cells, while follicular B cells possibly produce more IFN-γ (47), suggesting that they have a different effect on the polarization and regulation of T cells. Some of the B cells, for example those that induce IL-4 secretion by T cells via OX40L, require stimulation with protein antigen (51), but some functions, such as the production of chemokines, only appear to require the stimulation of PRRs (50).

B cells are important for generating T-helper 1 and T-helper 2 cells in both the primary and secondary immune responses to infectious organisms (52, 53). However, it appears that different B cell signals are required for the primary and secondary response. The primary immune response requires only signals via TLR-induced secretion of cytokines by B cells, but the secondary immune response requires BCR-mediated antigen presentation (52).

The studies illustrate that B cells are a diverse group of cells with multiple different functions, antibody-dependent and antibody-independent, that are important for the primary and/or secondary immune response.

T cells

T cells are a group of lymphocytes that recognize MHC-bound peptide. $CD4^+$ T cells are only able to recognize peptides bound to MHC class II molecules, which are expressed by APCs, such as macrophages, DCs or B cells, whilst $CD8^+$ T cells are only able to recognize peptides bound to MHC class I molecules, which are expressed by most nucleated cells (54). They can induce an immune response by activating and recruiting other leukocytes, induce B cell differentiation and antibody synthesis (55, 56) or induce cell lysis (57). They can also suppress the immune response (58).

On expressing GATA-3 and Notch-1, the CLP becomes committed to the T cell lineage (30). The pro-T cell migrates to the thymus to undergo maturation. There are a number of stages to the maturation process. The first stage involves the rearrangement of the TCR and the expression of the CD4 and CD8 co-stimulatory molecules followed by positive selection of T cells that are able to recognize self-MHC molecules with low avidity but deletion of cells that recognize antigens with high avidity. Finally the T cells lose either CD4 or CD8 to commit them to becoming either part of the $CD4^+$ helper or regulatory T cell subset (that recognize peptide on MHC class II molecules) or the $CD8^+$ cytotoxic T cell subset (that recognize peptide on MHC class I molecules), before they then migrate into the periphery. At this stage, when the T cells have not yet encountered antigen presented by an APC in the periphery, the

T cells are considered naïve. When antigen is presented with appropriate co-stimulation the T cell then differentiates into an effector or regulatory cell.

$CD4^+$ T helper cells

To date a number of different $CD4^+$ effector (or helper) T cell subsets have been described. They are all defined by the expression of the IL-7 receptor (CD127) but they express different chemokine receptors (Table 1.1.2) (59). Each subset has a different cytokine profile; T helper 1 (T_H1) cells are characterized by the expression of IFN-γ and IL-2, T_H2 cells by IL-4, 5 and 13 and T_H17 by IL-17, 21 and 22 (60). During infection these cytokines recruit and stimulate other cells, including B cells, neutrophils and macrophages to mount an appropriate immune response. T_H1 cells act against intracellular pathogens by recruiting macrophages, neutrophils and stimulating B cells to produce opsonizing and complement-fixing IgG antibodies. T_H2 cells respond to helminthic infections by activating eosinophils and stimulating B cells to produce IgE. Less is known about T_H17 cells but they are thought to induce immune responses to extracellular pathogens by recruiting neutrophils. There is also a subset of T cells, called T follicular helper cells (T_{FH}), which reside in the germinal centres in SLOs. These cells provide signals to germinal centre B cells, which induce their differentiation into long-lived memory B cells and antibody secreting plasma cells (61) that then home to survival niches in the bone marrow (62). The large number of different subsets illustrates the wide range of responses that the immune system can mount to different pathogens.

	Naïve	Central memory			Effector/ Revertant memory		
		Non-polarized	Pre- T_H1	Pre-T_H2	T_H1	T_H2	T_H17
CD27	+	+	+	+	–	–	–
CD28	+	+	+	+	+/–	+/–	+/–
CCR7	+	+	+	+	–	–	–
CD45RA	+	–	–	–	–/+	–/+	–/+
CD57	–	–	–	–	–/+	–/+	–/+
CD11a	low	high	high	high	high	high	high
CXCR3			+		+		
CXCR5		+					
CCR3						+	
CCR4				+		+	+
CCR5					+		
CCR6							+

Table 1.1.2 – Surface markers of human $CD4^+$ T cells
(59)

Regulatory T cells

Regulatory T cells play a key role in maintaining tolerance to self-antigen and preventing autoimmunity. They work through a variety of mechanisms, including suppressing or inducing cell death of effector T cells and APCs. They may also suppress differentiation of B cells into plasma cells and the production of antibodies. They are characterized by the expression of the intracellular signaling molecule forkhead box-P3 (FoxP3), and several surface markers, including the IL-2 receptor (CD25), cytotoxic T-lymphocyte antigen 4 (CTLA-4) and the glucocorticoid-induced TNFR-related (GITR) protein (63).

T helper cell differentiation

The polarization of T_H cells toward one or other subset is determined by a number of different factors, including the duration of antigen presentation (64), co-stimulatory factors (51, 65), and exposure to specific combinations of cytokines (60). T_H1 cells require IFN-γ and IL-12,

T_H2 cells require IL-4 and IL-2, T_H17 cells require transforming growth factor beta (TGF-β) and IL-6 and regulatory T cells require TGF-β and IL-2. Although it is thought that DCs are the main source of these signals, and therefore the cell that mediates T cell activation and differentiation (66), other cells, such as B cells, might also play an important role (44, 45, 47, 52, 53, 67).

1.1.6 Lymphocyte migration

The diverse nature of the adaptive immune system requires lymphocytes to survey both the lymphoid and non-lymphoid tissue and induce an effector response, either by recruiting more cells to an infected site or producing antibody, when a pathogen is detected. The process of migration between lymphoid organs and from lymphoid organs to non-lymphoid tissue is called lymphocyte recirculation. Both B and T cells recirculate, although there are differences. T cells, for example, appear to be more efficient at migrating to peripheral lymphoid tissue, whilst B cells preferentially migrate to the spleen and to Peyer's patches in the intestines (68, 69).

Naïve T cells continuously migrate through the blood stream into secondary lymphoid tissue and back into the blood stream until they encounter antigen on an APC. If presented with an antigen in a lymph node, the T cells differentiate into effector memory T cells and then migrate back into the blood stream. From the blood stream the effector T cells are then directed towards the infected or inflamed tissue (called lymphocyte homing). The blood vessels are therefore the main point of origin from which effector T cells enter infected or inflamed tissue.

To move within the lymph nodes, naïve T cells express high levels of L-selectin (CD62L) and CCR7, which are recruited by CCL19 and CCL21. After encountering antigen these receptors are downregulated. In order to be guided towards the infected area, the T cells express chemokine receptors specific for the infected tissue and the cells in the infected tissue express the corresponding recruiting chemokine. The skin, for example, recruits cutaneous memory T

cells by expressing the thymus and activation chemokine (TARC) that binds to CCR4, which is expressed on the T cells. However, once the T cell reaches the infected or inflamed tissue it needs to attach itself to the blood vessel and pass between the endothelial cells in order to enter the tissue parenchyma. This is a complicated process that requires the expression of a number of different adhesion molecules (integrins), mainly lymphocyte function-associated antigen 1 (LFA-1) or very late antigen 4 (VLA-4), by the T cell and corresponding ligands by the endothelial cells (12).

It is common to see $CD4^+$ and $CD8^+$ T cells in the non-lymphoid tissue of healthy individuals, predominantly surrounding the blood vessels, with macrophages scattered within the tissue. The T cells are phenotypically memory cells and show evidence of activation. However it is not certain whether these T cells reside within the tissue or actively circulate through the tissue and return to the SLOs. Studies suggest that $CD4^+$ T cells move quickly through the tissue, whilst $CD8^+$ T cells move more slowly and possibly reside for long periods (70, 71). However, evidence suggests that $CD4^+$ T cells initiate the formation of ectopic lymphoid tissue (ELT), showing that with the appropriate signals some $CD4^+$ T cells will remain in the non-lymphoid tissue (72). How frequently $CD4^+$ T cells recirculate and what signals are necessary before they commit themselves to stay in the tissue remain uncertain.

Less is known about the mechanisms that control mature B cell migration. Naïve B cells recirculate through non-lymphoid tissue like T cells (73), but unlike naïve T cells they need to undergo a second round of antigen receptor editing, which requires help from additional leukocytes. Therefore, B cells preferentially migrate to specialized microenvironments within SLOs, which is partly mediated by the binding of CXCR4, expressed on B cells, to the chemoattractant, stromal cell derived factor one-alpha (SDF-1α) (74). When B cells encounter antigen, they then differentiate into antibody-secreting cells (ASC) or memory B cells. Both IgA and IgG secreting cells migrate to the bone marrow, but IgA secreting cells also migrate to mucosal surfaces, whilst IgG secreting cells migrate to sites of tissue inflammation (75). Memory B cells appear to migrate to and/or remain in the spleen. However, the presence of a

small number of memory B cells in people who have had splenectomies suggests that there are additional minor storage sites (76). During infection, memory B cells are recruited by the chemokine CXCL13, which is secreted by monocytes (77), and produce IgG locally in ELT (78). The studies therefore suggest that antibodies can get into the non-lymphoid tissue via three sources; from ASCs in the bone marrow that secrete antibodies into the circulation, via infiltrating ASCs that migrate from SLOs or from ASCs that arise from memory B cells in ELT.

Each organ is likely to have differences in how lymphocytes recirculate. The lungs of mice, for example, have developed specialized lymphoid tissue that utilizes a distinctive combination of adhesion molecules to retain lymphocytes and provides a microenvironment where lung-specific cells can present antigen to naïve T cells (79). The brain, however, does not have any lymphoid tissue or lymphatic vessels. Therefore, the only entry and exit points for lymphocytes are via blood vessels. Antibody is also present at much lower levels in the cerebrospinal fluid than in the circulation suggesting that it does not pass through the barriers of the central nervous system easily. There is tight control of the migration of lymphocytes through the brain but the mechanisms are not fully understood. Expression of specific adhesion molecules, chemokines and chemokine receptors by both endothelial cells and lymphocytes have been shown to be important (80, 81). Whether cell migration depends on antigen specificity is less clear. One study has shown that T cell entry into the brain is not antigen specific but the cells are retained in the tissue if they recognize brain antigen, suggesting that there is careful selection of antigen specific T cells inside the brain (82). Other studies show that T cells require an antigen-specific signal to cross the blood brain barrier (83, 84).

B cell migration into the brain has not been studied as extensively as T cells. One study has shown that murine memory B cells from SLOs can locate antigen in the brain and generate an intrathecal humoral immune response (85). This local production of antibody by infiltrating B cells appears to be important for the clearance of acute infections (78, 86). It is difficult to

investigate the migration of B cells in human brains but one study has shown that they migrate across brain endothelium more rapidly than T cells, possibly due to differences in their responsiveness to specific chemokines (87).

1.1.7 Summary

This section has described the development of B and T cells, their multiple functions and interactions with each other, and how they both mount effector responses to pathogens, by secreting antibodies and/or differentiating into tissue infiltrating effector cells. The immune response starts with the primary adaptive immune response, which consists of distinct phases; formation of a naïve lymphocyte pool (lymphopoiesis), antigen recognition, clonal expansion and differentiation (activation), antigen elimination, contraction (homeostasis) and then finally memory (1). The secondary (recall) immune response following re-exposure to antigen is similar but more rapid and larger. They have another important difference, which is that in the primary immune response naïve lymphocytes mostly recirculate within the SLOs, whilst in the secondary immune response memory lymphocytes mostly recirculate within the non-lymphoid tissue, although a subset of memory lymphocytes can remain resident in niches within SLOs (88).

Immune surveillance by recirculation is an important part of the adaptive immune response and therefore should be recognized as one of the phases, before antigen recognition. By including it the two immune responses it becomes easier to appreciate that the adaptive immune responses are dynamic, cyclical, interacting processes in 2 separate physical compartments (Figure 1.1.1). This model proposes that in the primary immune response naïve lymphocytes survey the SLOs and after recognizing antigen and clearing it, then provide a pool of memory lymphocytes, which survey the non-lymphoid tissue. In the secondary immune response if antigen is recognized in the non-lymphoid tissue the memory lymphocytes become activated and mount a more rapid immune response to clear the antigen. When the immune response contracts, the cells either die (26) or return to the memory lymphocyte pool (88).

The secondary immune response varies within the different types of non-lymphoid tissue because of their structural and functional differences and to protect them from specific environmental pressures. Understanding the mechanisms that control and regulate this process and how it differs within each tissue will help to understand how the immune system protects the body and also how immunological diseases occur.

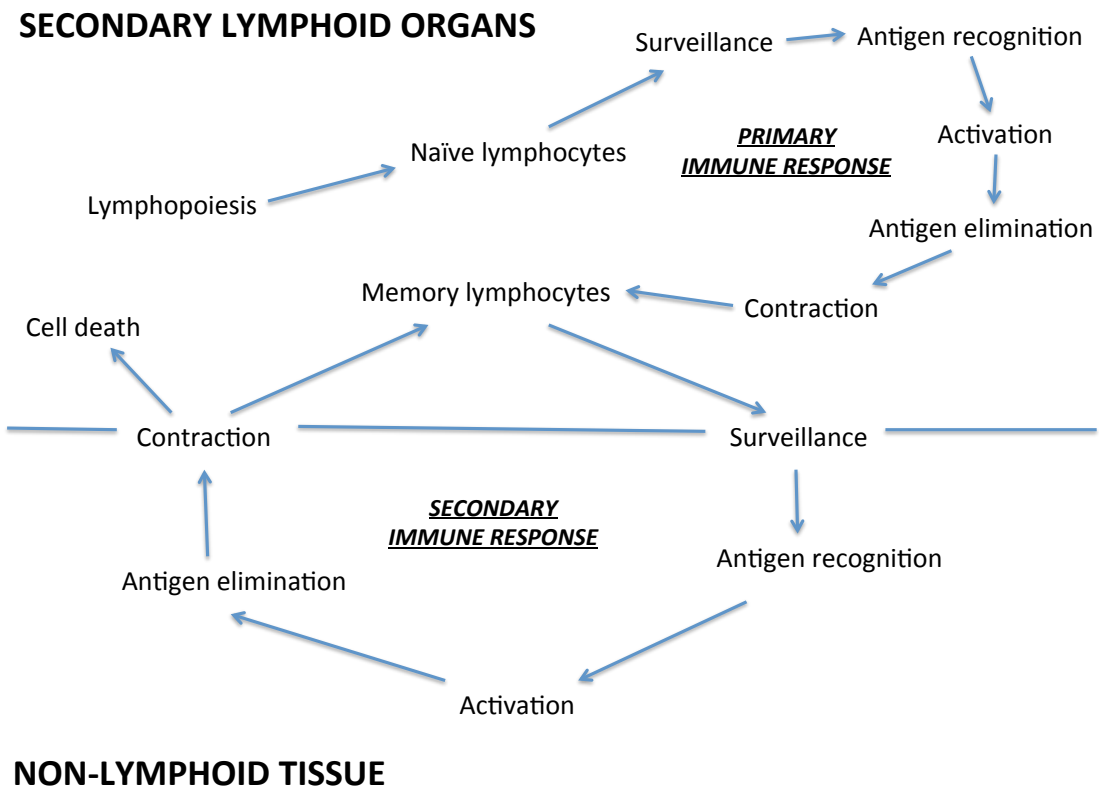


Figure 1.1.1 – Primary and secondary immune response
The secondary immune response is characterized by a pool of memory lymphocytes that are supplied by naïve lymphocytes that have been activated in the SLOs during the primary immune response. The memory lymphocytes mostly recirculate within the non-lymphoid tissue. After recognizing antigen they become activated (clonally expand) and eliminate the antigen. The immune response then contracts, either by the lymphocytes dying or returning to the memory lymphocyte pool where they continue to recirculate within the non-lymphoid tissue.

1.2.1 Diseases of the immune system

Diseases of the immune system can be due to either an insufficient immune response (immunodeficiency), self-directed damage due to an inappropriate innate immune response (autoinflammation) or an inappropriate adaptive immune response (hypersensitivity). Five types of hypersensitivity reactions have been described; type 1 (atopy) is due to IgE antibodies directed against common and normally harmless antigens (allergen), type 2 (cytotoxicity) is due to IgG or IgM antibodies directed against proteins on the cell surface, type 3 (immune-complex mediated) is due to IgG or IgM antibodies directed against soluble antigens, type 4 (delayed-type) is due to $CD4^+$ or $CD8^+$ T cells directed against an intracellular antigen and type 5 (receptor mediated) is due to antibodies that can either stimulate or inhibit cell receptors (89).

In some inflammatory diseases the pathological mechanisms can be explained by either autoinflammation or one of the hypersensitivity disorders. However, some inflammatory diseases appear to be due to a combination of these disorders. Crohns' disease, for example, has been shown to have features of both autoinflammation and Type 4 hypersensitivity (90). It has been proposed that these diseases are part of an immunologic disease continuum (91). These combinations might provide an explanation for the heterogeneous manifestations that are seen in some inflammatory diseases.

1.2.2 Autoimmunity and autoimmune disease

An immune response directed against self-antigen is referred to as autoimmunity. This process can be due to either autoantibodies or autoreactive T cells. It has been suggested that self-reactivity might be an important part of the normal immune response, in order to protect the body from malignancy (92), atherosclerosis (93) and infection (94). Some autoreactive $CD4^+$ T cells have been shown to protect the tissue from trauma induced-damage, i.e. they are important for repair and tissue homeostasis (95). However, autoantibodies and autoreactive T cells can induce hypersensitivity reactions (types 2 to 5). The point at which the recognition

of autoantigen leads to tissue damage and becomes detrimental to the host rather than protective is called autoimmune disease. There are two types of autoimmune disease; organ-specific, which are usually associated with autoantibodies that are directed against tissue specific antigen, and systemic, which are usually associated with autoantibodies that are directed against soluble antigens.

For autoreactive cells and antibodies to cause autoimmune disease additional events need to occur, either in sequence or in parallel. Sequential events that might need to occur include increased affinity of antibodies or the T cell receptor to antigen, immunoglobulin class-switching, complement activation and/or recruitment of other immune cells. Parallel events might include activation of other immune cells, such as macrophages and/or neutrophils, or changes within the target cell or tissue. A breakdown in tolerance, therefore, first occurs centrally, by failing to eliminate autoreactive B and T lymphocytes, and then peripherally, by failing to inhibit the autoreactive lymphocytes, recruitment of additional activated leukocytes and changes to the target cells, resulting in an immune response that causes a hypersensitivity reaction.

Interestingly, patients with immunodeficiency syndromes associated with B and/or T cell deficiency, such as X-linked aggammaglobulinaemia, combined variable immunodeficiency (CVID) (96) and acquired immune deficiency syndrome (AIDS) (97) are more susceptible to autoimmune disorders. This observation might suggest a causal link between infection and autoimmune disorders, but patients with immunodeficiency might also have reduced regulatory cells to maintain peripheral tolerance.

Most autoimmune diseases are characterized by the autoantibodies present in the blood rather than the T cell specificities, partly because of the relative ease with which the antibodies can be identified (typically by enzyme-linked immunosorbent assay (ELISA)). However, consideration also needs to be given to the T cell in the inflammatory process and research is needed to understand how it might contribute to the development of autoimmune disease.

1.3.1 Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a prototypic systemic autoimmune disease with a prevalence of 40 to 200 per 100,000. It has a female:male ratio of 6-12:1, mainly affecting women of child-bearing age. The prevalence varies in different ethnic and socioeconomic groups, being more common in Afro-Caribbean and Asian populations (98). It is characterized by the presence of antibodies directed against nuclear antigen, in particular against double-stranded DNA (dsDNA), histone complexes and ribonucleic acid (RNA)-binding proteins. All organs or systems can be affected, although the clinical manifestations vary markedly between patients. The disease course is variable and unpredictable with patients experiencing episodes of remission followed by relapse. There are certain environmental factors that are known to exacerbate the disease, such as exposure to ultra-violet light, but in most cases a precipitant cannot be established. The survival rates have improved considerably in the past 40 years as a result of the introduction of corticosteroids and other immunosuppressants, dialysis and renal transplantation but the disease still causes significant morbidity and increased mortality.

1.3.2 Clinical manifestations of SLE

Patients can present with a wide variety of different symptoms. Constitutional symptoms such as fever, weight loss and fatigue are common, but not specific to the disease. Likewise joint and muscle pain (with or without inflammation) and sicca symptoms are also common, but these symptoms can occur in several other autoimmune diseases. The organs that are most frequently affected in SLE are the kidneys, skin and joints, but no organ is protected. Most organ-affected manifestations are due to inflammation (or hypersensitivity) but they can also be due to vascular occlusion by thrombi (99) or atherosclerosis (100).

The diverse nature of the disease and non-specificity of some of the abnormalities seen in the disease means that there is no single symptom, sign or serological factor that is seen in all patients that can alone be used to diagnose SLE. However, classification criteria for SLE have

been devised to ensure that there is a consistent definition of the disease for the purposes of research and surveillance. These classification criteria require a combination of objective clinical signs from commonly affected organs and/or serological or haematological abnormalities to be present. The American College of Rheumatology (ACR) revised criteria for the classification of SLE was the most widely used until the Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE replaced it in 2012 (101, 102). For a person to be defined as having SLE the ACR revised classification criteria required at least four out of 11 criteria to be present (Table 1.3.1) (101), whereas the SLICC classification criteria requires fulfillment of at least four out of 17 criteria, with at least one clinical criterion and one immunologic criterion, or lupus nephritis in the presence of ANA or anti-dsDNA antibodies (Table 1.3.2) (102).

Whilst it is a disease that can affect all organ systems it has certain symptoms which make it more distinguishable from other systemic autoimmune diseases, such as rheumatoid arthritis, primary Sjögren's syndrome and sytemic sclerosis, even though they share some of the serological abnormalities (103). These symptoms include malar rashes, mouth ulcers, cerebral involvement and glomerulonephritis. Most studies have focused on understanding the pathogenesis of glomerulonephritis because it is one of the more common disease manifestations and it is associated with higher mortality and morbidity, particularly if it is untreated, making it more of a priority for research (104). It is also easier to get histological samples from the kidneys for analysis compared to other sites of inflammation, such as the mucous membranes and brain. However, cluster analyses suggest that certain symptoms are less likely to be associated with glomerulonephritis (105, 106) and therefore it cannot be assumed that the factors that cause glomerulonephritis are the same as those that cause other symptoms.

Incomplete lupus erythematosus

Multiple symptoms and serological abnormalities are considered a defining feature of the disease as illustrated by the ACR and SLICC classification criteria. However, disease usually

starts with the stepwise accumulation of autoantibodies that are typically seen in the disease and then with the development of one of the ACR criteria symptoms before the diagnosis is made (107).

Some patients do not develop enough clinical manifestations and serological abnormalities to fulfill the ACR or SLICC classification criteria for SLE. These patients are sometimes diagnosed as having an undifferentiated autoimmune rheumatic disease (or connective tissue disease) or incomplete lupus erythematosus (ILE). Studies show that the percentage of patients with ILE that eventually develop SLE ranges from 3 to 57% over a mean follow-up time of 1.6 to 13 years (108-112). The large differences in the percentage of patients with ILE that transformed into SLE between studies is likely to be partly due to the differences in the duration of follow-up, suggested by an 8 year prospective study in a Danish community that found an estimated progression rate of SLE from ILE to be 3.64 per 100 person-years at risk (113).

The most common symptoms in patients with ILE were photosensitivity (23-42%), arthritis (10-47%), malar rash (4-21%) and serositis (1-35%). Two out of five studies showed that malar rash is a risk factor for SLE progression. One of these two studies also showed that mouth ulcers to be a risk factor. The other three studies did not identify any risk factors for SLE progression from the baseline symptoms.

Criterion	Definition
Malar rash	Fixed erythema over the malar eminences, tending to spare the nasolabial folds
Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions
Photosensitivity	Skin rash that occurs following exposure to sunlight
Oral ulcers	Oral or nasopharyngeal ulceration
Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling or effusion
Serositis	a) Pleuritis – history of pleuritic pain or rub heard by physician or evidence of pleural effusion or b) Pericarditis – documented by ECG or rub or evidence of pericardial effusion
Renal disorder	a) Persistent proteinuria greater than 0.5g/day or greater than 3+ on urine dipstick or b) Cellular casts – may be red cell, haemoglobin, granular, tubular or mixed
Neurologic disorder	a) Seizures – in the absence of offending drugs or metabolic derangement or b) Psychosis – in the absence of offending drugs or metabolic derangement
Haematologic disorder	a) Haemolytic anaemia – with reticulocytosis or b) Leukopaenia – less than 4 x 10^9/L on two or more occasions or c) Lymphopaenia – less than 1.5 x 10^9/L on two or more occasions
Immunologic disorder	a) Anti-DNA – antibody to native DNA in abnormal titre or b) Anti-Sm – presence of antibody to Sm nuclear antigen or c) Antiphospholipid antibody – either an abnormal serum level of IgG or IgM anticardiolipin antibodies, a positive test result for lupus anticoagulant using a standard method, or a false serologic test result for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test
Antinuclear antibody	An abnormal titre of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome
For the purpose of identifying patients in clinical studies, a person is defined as having SLE if any 4 or more of the 11 criteria are present, serially or simultaneously, during any interval of observation	

Table 1.3.1 – American College for Rheumatology revised criteria for the classification of SLE
(101)

Clinical Criteria	Immunologic Criteria
Acute Cutaneous Lupus Chronic Cutaneous Lupus Oral or Nasal ulcers Non-scarring alopecia Arthritis Serositis Renal Neurologic Haemolytic anaemia Leukopaenia Thrombocytopaenia	ANA Anti-DNA Anti-Sm Antiphospholipid antibodies Low complement (C3, C4, CH50) Direct Coombs' test (do not count in the presence of haemolytic anaemia)
Requirements: >4 criteria (at least 1 clinical and 1 laboratory criteria) OR biopsy-proven lupus nephritis with positive ANA or Anti-DNA	

Table 1.3.2 – Systemic Lupus International Collaborating Clinics classification criteria for SLE
(102)

1.3.3 Genetics and aetiology of SLE

Consistent with the heterogeneous nature of the disease, more than 50 genes, with a diverse range of functions, have also been associated with the disease (114). In a few cases patients have been found to have highly penetrant genetic mutations causing deficiency in complement proteins (115). However, the majority of cases of disease are due to genetic polymorphisms, some of which are still to be identified, that confer a susceptibility to disease (116, 117). The development of disease most likely requires a combination of these genetic polymorphisms.

All of the genetic associations that have been identified from the genome-wide analyses relate to immunological function or control of apoptosis. As is seen in other autoimmune diseases the strongest associations were found with the MHC class II molecules. Some additional genes that have been identified are linked to B and T cell function (Table 1.3.3). These genetic associations point at potential mechanisms that may be involved in the development and progression of the disease.

Genes are not the only factors that influence the development of SLE. Sex hormones are also likely to be important, because it is more prevalent in women, particularly during the second decade (118), and some studies have shown that there is an increase in disease flares during pregnancy (119). Environmental factors, such as infectious organisms (120), smoking (121), exposure to chemicals, such as silica and pesticides (121), and ultraviolet light (122), have also been implicated in the aetiopathogenesis of SLE. Drugs have also been shown to cause lupus-like diseases (123). The disease is, therefore, probably caused by a combination of genetic polymorphisms, hormones and environmental stimuli.

Gene	Odds Ratio	Function
The MHC	2.36, 2.01[*] 2.35[†]	Antigen presentation to T cells
BLK	1.39, 1.22[*] 0.69[†]	BCR signaling
BANK1	1.38[*] 1.31[†]	BCR signaling
LYN	1.30[*] 0.77[†]	BCR signaling
FCGR2B	1.21, 1.33[*] - [†]	Inhibits B cell activation
CD40	- [*] 0.63[†]	Synergizes with BCR signaling pathway
IL10	- [*] 1.19[†]	B cell stimulant
STAT4	1.53, 1.50[*] 1.55[†]	T cell differentiation
PTPN22	1.53, 1.49[*] 1.4[†]	TCR signaling
ITGAM	1.62, 1.33[*] 1.62[†]	Leukocyte adhesion
TNFSF4	- [*] 1.46[†]	Co-stimulatory signal by APCs to $CD4^+$ T cells

Table 1.3.3 – Genetic polymorphisms associated with lymphocyte function in SLE
[*](116), [†](117)

1.3.4 Clinical assessment

One of the many difficulties of treating patients with SLE is determining whether the symptoms that the patient is experiencing is due to an active inflammatory process, damage (i.e. scarring) from past inflammation or due to a pathology unrelated to SLE. An objective measure of active disease is important for deciding when to initiate or increase therapy, for establishing the efficacy of a therapy, both for the individual patient in the clinic and in clinical trials, and also for understanding whether an immunological abnormality has a role in the inflammatory process or is secondary to tissue damage. However, as yet there is no single, commercially available assay that can detect active inflammation with 100% sensitivity and specificity. Nevertheless the appearance or worsening of a number of symptoms and signs has been consistently shown to suggest an active inflammatory process that, if left untreated, would ultimately cause damage to the tissue and increase mortality or morbidity for the patient. Several different clinical assessment tools have therefore been designed, each by consensus between investigators, to agree on criteria for active disease using a combination of these symptoms and signs. These assessment tools, which include the European Consensus Lupus Activity Measure (ECLAM), the Systemic Lupus Activity Measure (SLAM), the Systemic Lupus Disease Activity Index (SLEDAI) and the British Isles Lupus Assessment Group (BILAG) index, have been validated and found to be reliable and sensitive, but they all differ between each other, both in terms of the logistics of the assessment process and in terms of their strengths and limitations. The BILAG index, for example, provides a global score for disease activity and organ-specific scores, to allow for differences in drug efficacy, and possibly pathology, between organ systems. Other indices incorporate scores for treatment and immunological variables, such as complement levels (Table 1.3.4) (124).

The role of histological analysis in assessing disease activity is controversial. In animal studies of SLE, histological assessment of the kidneys, skin or joints is usually performed to show the presence of inflammation. However, human disease is more heterogeneous, which is demonstrated by the wide variation in histological abnormalities that have been reported in

the biopsies of both kidneys and skin (125, 126). This variation can be due to differences in the stage of the disease, the disease duration, the cause of the inflammation and the presence of tissue damage. Certain abnormalities are considered markers of disease activity, which in renal disease can be used to form an activity index. However, using histology as a regular disease assessment tool is impractical, and therefore serological factors and disease assessment indices using symptoms, signs and laboratory data remain the most widely used in clinical studies. Nevertheless, the information that tissue histology provides is extremely useful for making a diagnosis, staging, predicting outcomes and guiding treatment, especially in kidney disease. It is also very useful for understanding the pathogenesis of the disease.

	No. of items/ Content (organ systems)	**Recall period (days)**	**Strengths**	**Limitations**
BILAG	101 items + 5 items for GFR/ 9 organ systems	28	Can detect change in disease activity over time Can show activity in each system Records flare and response Based on physician's intention to treat	Formal training of raters needed Computer required to calculate the score Higher respondent and administrative burden
SLEDAI-2K	24 items/ 9 organ systems	30	Easy to use Scoring is simple additive	Does not record improving or worsening disease Does not assess severity of each organ system
SLAM-R	9 items/ 9 organ systems + 7 laboratory features	30	Includes both disease activity and disease severity	Scoring can be subjective Difficulty in distinguishing changes
ECLAM	33 items/ 10 organ systems + ESR and complement levels	30	Easy to use Scoring is simple additive	Formal training of raters needed Global score only – will miss changes in severity over time

Table 1.3.4 – Comparison of physician completed disease activity indices in SLE (124)

1.3.5 Biomarkers for SLE disease activity

A biomarker is a gene or biological molecule whose quantifiable change correlates with the disease process and/or its manifestations. Given the abnormalities that are seen in the tissue of patients with SLE, disease biomarkers can be divided into four different types; those associated with humoral dysfunction, immune complex deposition, cell migration and tissue

damage. These biomarkers vary in their specificity and sensitivity to active disease. In general the humoral biomarkers, such as anti-dsDNA and Sm antibodies are the most specific for SLE but are not very sensitive to changes in disease activity (127). An alternative biomarker of humoral dysfunction is the plasma cell count, which has been shown to correlate with disease activity, and was independent of changes in anti-dsDNA antibody levels (128). Direct measurement of immune complexes has proved challenging due to high false positive results in patients with SLE (129). Decreases in complement levels, notably C3 and C4, are often used as an indirect measure of immune complex formation (and subsequent clearance), although they might also reflect direct complement deposition in the tissue. However, complement levels vary between individuals, sometimes rising during active disease or before a clinical flare (130), and sometimes being low due to a hereditary deficiency (131). A decrease in complement proteins has been shown to correlate with disease activity in patients with moderate to severe disease but not mild disease, particularly patients without renal disease (130). Patients with active disease but normal complement levels might also have normal anti-dsDNA antibody levels but follow-up of these patients show that the markers might appear at a later stage of the disease (132).

Various cytokines, soluble cytokine receptors, cell surface markers and adhesion molecules have also been studied as candidate biomarkers of active disease. The percentage of HLA-DR^+ T cells in the circulation might also be useful for predicting flares (130). Levels of adhesion molecules, chemokines and T cells in the urine have been correlated with active nephritis and therefore might be useful biomarkers for renal disease (130, 133, 134).

Circulating endothelial microparticles have been detected in patients with persistent endothelial damage despite the disease appearing to be under control (135) and have also been detected in patients with atherosclerotic disease (136), which patients with SLE are predisposed to. They might therefore be useful for detecting sub-clinical inflammation in SLE.

Studies have also tried to find biomarkers that might predict specific clinical manifestations. Most studies have focused on the predictive value of autoantibodies. There is agreement that antibodies against nucleosomes and C1q are useful for predicting renal disease (137). Some autoantibodies might predict thrombosis (138), cytopaenias (139), cerebral disease (137) and xerostomia (140). However, there are several symptoms that do not appear to have strong associations with autoantibodies. Correlations between abnormalities of peripheral blood leukocytes and clinical symptoms have not been studied as extensively as autoantibodies and are therefore not currently considered useful biomarkers for predicting disease symptoms.

One of the problems with developing disease biomarkers for SLE has been that the pathogenesis is likely to be due to a combination of factors, with the contribution of each factor varying between individuals. Therefore, the ideal biomarker is most likely going to be a composite of several factors that are part of all or most of the pathological processes causing the disease in that individual.

The most likely reason for the difficulty in developing reliable clinical assessment tools and finding biomarkers that could be used in SLE is that clinicians assume that the pathogenesis is identical in all patients. However, the heterogeneity of the disease suggests that the underlying pathology differs between patients. A better understanding of the pathogenesis of the disease is likely to lead to improvements in the reliability of clinical assessment tools and biomarkers.

1.3.6 Therapeutic management of SLE

The main treatment options for patients with SLE include corticosteroids, anti-malarial and cytotoxic agents, which include azathioprine, methotrexate, mycophenolate mofetil and cyclophophosphamide (141). Corticosteroids and cytotoxic agents are associated with more side effect and long-term complications, in particular a higher risk of infection and malignancy. Therefore, the choice of medication depends on the severity of the disease, which is usually determined by the degree of internal organ involvement. For musculoskeletal

and cutaneous symptoms, the treatment would typically start with anti-malarial agents and low-dose corticosteroids when needed. For internal organ disease, in particular glomerulonephritis, vasculitis and cerebral disease, the treatment would usually require the addition of cytotoxic agents and high-dose corticosteroids. The side effects of cytotoxic drugs are a result of their broad range of action. Therefore, more targeted therapies have been developed, which have fewer side effects (Table 1.3.5). These drugs target surface molecules on B and T cells, either to deplete them from the circulation or to modify their function, and cytokines, such as BAFF and IFN-α. However, despite positive results in open-labeled studies, the efficacy of these targeted therapies have shown disappointing results in randomized controlled trials, with the possible exception of Belimumab, a drug that inhibits BAFF, which showed statistically significant benefits, although only in one of the two time-points (142).

The treatment of moderate to severe disease is often divided into induction and maintenance therapy (143). Induction therapy typically involves giving intensive immunosuppression in order to stop the inflammatory process as rapidly as possible with the aim of preventing further damage to the affected organ. During maintenance therapy the immunosuppression is reduced in order to prevent long-term complications from the drugs. Whilst the treatment regimen is usually guided by clinical trials, it might need to be tailored to the individual patient depending on other factors, such as the severity of the disease or co-morbidities that the patient might have.

There have been significant improvements in the mortality rates over the last 40 years (144), but only anti-malarial agents have been shown to improve the long-term survival of patients with SLE (145). In addition, whilst several new drugs have been trialled in SLE only one has been licensed for the treatment of SLE in the last 30 years. The failure of trials to show convincing efficacy is likely to be due to a combination of factors, including the design of the trials, such as inclusion criteria, excessive concomitant therapy, notably corticosteroids, the use of therapy in the comparator arm, defined end-points, the choice of assessment tool and

the length of follow-up (146). However, in a disease with diverse clinical manifestations due to a complex pathological process, a drug might be effective with one manifestation of the disease but not another. This possible explanation becomes more important as the therapeutic target increases in specificity. To illustrate this point, a secondary analysis of a recent clinical trial with a drug targeting CTLA-4 co-stimulation showed statistically significant improvements in arthritis despite not demonstrating an improvement in the disease overall (147). Likewise, certain drugs have been shown to only lead to significant benefits in patients with specific antibody profiles (148, 149). It could therefore be argued that until the pathogenesis of the disease and in particular the reasons for the heterogeneous manifestations of the disease are understood, the drugs that have the broadest mechanism of action will be the most likely to demonstrate efficacy in SLE. In other words as the targets become more specific, the clinical trials will need either greater numbers of patients or selection of patients with specific clinical or immunological characteristics in order to demonstrate therapeutic efficacy.

Drug	Mechanism of action	Targets	Efficacy
Prednisolone	Inhibition of NF-κB	All leukocytes	Effective for most SLE manifestations. Licensed for SLE
Hydroxychloroquine	Inhibits TLR signaling	Monocytes Dendritic cells B cells	Effective for cutaneous LE, arthritis and constitutional symptoms. Licensed for SLE
Cyclophosphamide	DNA alkylation leading to cell death	Bone marrow (leukopoiesis) All leukocytes	Effective for most severe SLE manifestations, particularly nephritis
Azathioprine	Inhibits DNA synthesis	Bone marrow (leukopoiesis) All leukocytes	Effective for lupus nephritis. Less effective than cyclophosphamide
Mycophenolate Mofetil (MMF)	Inhibits purine biosynthesis by lymphocytes	B cells T cells	Effective in lupus nephritis. Comparable with cyclophosphamide.
Rituximab	B cell depletion via ADCC/apoptosis	B cells	Efficacy not demonstrated in RCT
Belimumab	BAFF blockade	B cell activation	Efficacy demonstrated in RCT at 52 weeks only
Abatacept	Binds to B7 on APCs	T cell activation	Only effective for arthritis
Abetimus Sodium	Binds to anti-dsDNA antibodies	Anti-dsDNA antibodies	Efficacy not demonstrated in RCT

Table 1.3.5 – Therapeutic options in SLE
(141, 147, 149, 150)

1.4.1 Pathogenesis of SLE

There is evidence of at least two hypersensitivity reactions in patients with SLE. Firstly, type 2 hypersensitivity reactions are seen in a group of patients who develop autoimmune haemolysis and thrombocytopaenia. However, this possibly accounts for only a small proportion of the disease manifestations. Most patients develop some organ involvement, which has been commonly attributed to immune complex mediated disease, i.e. type 3 hypersensitivity reactions, particularly in lupus nephritis. Antibodies to dsDNA are thought to be an important cause of this immune complex deposition (151). However, many of these manifestations are also associated with the cellular infiltrates, most commonly T and B cells, macrophages and, occasionally, neutrophils (126, 134, 152-155). The presence of these cells, particularly T and B cells, do not fit into the classical type 3 hypersensitivity reaction, and

their exact role in the pathogenesis of the disease is not very well understood. However, they are associated with poor outcomes suggesting that they are contributing to the damage that occurs in the tissue.

Vasculitis in SLE – Arthus or Schwartzman reaction?

Two studies have shown that the incidence of vasculitis in patients with SLE is between 11 and 33% (156, 157). The vasculitis was cutaneous in over 80% of cases. Internal organs that were affected included nerves, digits, retina and mesentery. One study showed that the vasculitis involved small vessels in 86% of cases and medium-sized vessels in 14% of cases (157). Both studies found an association with lymphopaenia and anti-phospholipid syndrome or the presence of antiphospholipid antibodies. Hypocomplementaemia was associated with vasculitis in one of the studies (157). Neither study found an association with glomerulonephritis.

Immune complex deposition leading to activation of complement (Arthus reaction) is one of the possible causes of vasculitis, but circulating or deposited immune complexes are not always detected in patients with vasculitis. It is also possible that autoantibodies directly induce endothelial cell death, although they have only been shown to induce death by apoptosis rather than cell lysis (158). A third explanation for lupus vasculitis has arisen from studies that have shown that several autoantibodies that have been found in patients with SLE can induce endothelial cells to express adhesion molecules, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) (159-161). Rather than causing a type 2 or type 3 hypersensitivity reaction, the autoantibodies might instead be priming endothelial cells to bind and recruit activated leukocytes, causing a cell mediated immune response (Schwartzman reaction) (162, 163). Proteolytic enzymes released by neutrophils and $CD8^+$ T cells would be the likely cause of endothelial cell necrosis in this process. Observations that T cells and neutrophils from patients with vasculitis express increased levels of the adhesion molecules that bind to ICAM-1 and VCAM-1 support this hypothesis (164, 165).

1.4.2 Histological studies

Although the disease does not start in the peripheral tissue, this is nevertheless where the autoimmune response exerts its effect and ultimately induces damage. A large amount of information can therefore be obtained from careful analysis of the tissue. The difficulty with histological analyses is that the disease can affect different organs in each patient and have multiple different effects within each organ. However, there are a number of similarities, which provide important information about the inflammatory process that causes the disease.

The most commonly studied tissues from patients with SLE are those from the skin and kidneys. Immunofluorescence studies have identified deposits of IgM, IgG, IgA, complement factors, such as C3, and DNA in the tissue (166, 167). Deposition is mostly seen in areas where there is a basement membrane, such as blood vessel walls (168, 169), the dermal-epidermal junction (167), renal tubules and glomeruli (166, 169, 170), possibly because the basement membrane can trap nucleic acid (171). In glomerular lesions IgG is the most common class of antibody present (170), but in skin lesions the most common class present is IgM, although disease severity is greater when IgM is present with IgG and/or IgA (167). Immune depositis with IgM, IgG and/or IgA are also seen in non-lesional skin (168), which raises questions about their relevance in the disease process and the value of using immunofluorescence as a prognostic tool in SLE.

Lymphocytes, particularly T cells, are the most common leukocyte to be seen in renal and skin lesions, either in a perivascular distribution or in the interstitium. Granulocytes, in particular macrophages and neutrophils, are also commonly seen in the tissue (126, 172). T cells, either in the renal tissue or urine, correlate with active nephritis, increased renal damage and impairment (134, 154, 155, 173). The lungs and heart have been less well studied due to fewer biopsies being taken from patients but similar results have been seen with immune deposits along the blood vessel wall and perivascular and interstitial leukocytic infiltrates (174, 175).

In addition to the inflammatory changes, abnormalities in the tissue parenchyma also provide some information about the disease process. In the skin and glomeruli, characteristic changes of the epithelial and endothelial cells can be seen, but changes can also be seen in tissue specific cells, such as the mesangial cells in the glomerulus and the dermal cells in the skin. Active disease is often characterized by cell necrosis, but might show other features that are specific for that tissue, such as crescents in the glomeruli, whilst chronic disease is indicated by fibrotic or sclerotic changes and tissue atrophy (126, 176).

Characteristic signs can be absent from affected tissue (126). Likewise, abnormalities, such as immune deposits, can be found in non-lesional skin, although might still correlate with active non-cutaneous disease (177). It is, therefore, difficult to make a clear causal link between the inflammation and damage that occurs in the tissue, which then makes it difficult to identify biomarkers and formulate effective treatment strategies for patients.

1.4.3 Humoral abnormalities in SLE

The presence of autoantibodies is considered the central immunological abnormality in SLE. They are produced by plasma cells, which differentiate from autoreactive B cells. Anti-nuclear antibodies (ANA) are the most common antibodies, found in over 95% of patients. In SLE, these antibodies most commonly bind dsDNA, histone complexes, which bind to DNA and RNA-binding proteins. In addition, antibodies can also be found that bind soluble molecules, such as those that regulate the complement and coagulation cascades, rheumatoid factor and phospholipids, as well as antigens found on the surface of cells. Several of these autoantibodies can also be found in other systemic and organ-specific autoimmune diseases but certain antibodies, such as those that bind dsDNA and the RNA-binding protein, Sm, are highly specific for SLE (178).

Most studies have focused on the direct pathogenic effects that autoantibodies have on the tissue parenchyma, in particular their deposition as immune complexes within the tissue. However, antinuclear and antiphospholipid antibodies appear in the blood of patients many

years before diagnosis or the first symptom. Typically, antibodies to the RNA-binding proteins, Ro and La, appear the earliest, followed by antiphospholipid antibodies, then anti-dsDNA antibodies and then anti-Sm antibodies (107). In addition, with the possible exception of a few autoantibodies, most do not perform very well as biomarkers (127). Many explanations have been provided for the poor correlation between autoantibodies and active disease, but the most likely explanations are that either certain factors are required in addition to the immune complexes and/or that the autoantibodies exert an effect upstream of the inflammatory cascade, which would mean that it might take many months or years for the subsequent tissue inflammation and damage to become clinically apparent.

Role of autoantibodies in SLE

As previously mentioned, antibodies can exert multiple effects. Autoantibodies in SLE, have shown that they are able to induce antibody-dependent cell mediated cytotoxicity (type 2 hypersensitivity) and immune complex mediated cytotoxicity (type 3 hypersensitivity), but have also been shown to directly stimulate APCs and upregulate adhesion molecules on endothelial cells, suggesting that they might also have a role in cell mediated cytotoxicity (type 4 hypersensitivity). They also remove molecules important for the normal clearance of apoptotic bodies.

Several autoantibodies that are seen in SLE have been shown to have multiple pathogenic effects. For example, anti-C1q antibodies can bind to and inhibit important molecules that regulate the clearance of apoptotic bodies, but have also been shown to form immune complexes and deposit in the glomerular basement membrane (179). Likewise, immune complexes containing dsDNA or nucleosomes can deposit in the glomerulus (180) and have also been shown to stimulate DCs, via the ligation of Fc receptors and TLR-9, to produce high levels of the cytokine, interferon alpha (IFN-α) (181-184), which is thought to have an important role in systemic autoimmunity. They can also induce the production of other important inflammatory cytokines by both plasmacytoid DCs (PDC) and B cells (183, 185) and induce B cell proliferation and IgM synthesis in mice (186). These multiple functions

might explain why it is difficult to draw a clear connection between the autoantibody and the inflammatory lesions of active disease. Also, these autoantibodies need additional factors, such as antigen and leukocytes in order to exert their pathogenic effect, which might provide another explanation for why they do not correlate very well with active disease.

Anti-dsDNA antibodies

Antibodies directed against dsDNA can be found in healthy individuals, but these antibodies are typically specific for DNA from a given bacteria. In SLE, anti-dsDNA antibodies typically bind to a conserved DNA sequence (either the B or Z conformation) and cross-react with both bacterial and mammalian DNA (151, 187). These antibodies appear in the sera of approximately 60 to 83% of patients at some point during the course of the illness. The titres of anti-dsDNA antibodies correlate variably with disease activity but high titres have been shown to indicate an increased likelihood of clinical relapse (188). Furthermore the presence of anti-dsDNA antibodies has been shown to correlate well with glomerulonephritis (189). Several studies have shown the presence of anti-dsDNA antibodies in the glomerular eluates of patients with active nephritis (180). They have also been shown to deposit in the kidneys of experimental models of SLE (190-192)

It is unclear why anti-dsDNA antibodies are closely associated with glomerulonephritis but less clearly associated with other manifestations of SLE. There might be unique features of the glomerulus that encourages anti-dsDNA binding or immune complex formation. For example, it has been suggested that the specific localization of DNA-anti-dsDNA immune complexes in the glomerulus is due to a mechanism termed *in situ* immune complex formation. According to this mechanism the charge of the basement membrane of the glomerulus traps circulating nucleosomes with DNA, which then cause binding and accumulation of anti-dsDNA antibodies and subsequent complement deposition (193). Other possible explanations for anti-dsDNA induced glomerular damage include cross-reactive binding to a non-DNA glomerular antigen or direct penetration of glomerular cells by the antibodies (151).

It is possible that antibodies with different specificities induce similar mechanisms of inflammation in other organs but the unique function and structure of the glomerulus suggests that glomerulonephritis is a distinct pathological entity in SLE and not representative of the systemic disease process. Two animal models support this view. The first showed that certain anti-dsDNA antibodies when injected into the peritoneum of severe combined immunodeficiency (SCID) mice deposit in the glomeruli but not in other organs (194). A second animal model showed that when B cells were genetically modified so that their B cells could not secrete autoantibodies the mice did not get glomerular lesions, but still died prematurely with interstitial lesions (195). Not all anti-dsDNA antibodies induce glomerulonephritis. The reasons for this might relate to differences in the structure of the bound DNA, the presence of associated DNA-binding proteins, such as nucleosomes and histones, and the affinity of the antibody (196).

DNA has been shown to have another important immune property, which is the ability to stimulate leukocytes via TLR-9 stimulation. In doing so, DNA functions as a pathogen associated molecular protein (PAMP) to provide additional stimulation to cells during infection or stress (197). The DNA detected by TLR-9 is made up of unmethylated cytosine-phosphate-guanine (CpG) motifs, which is prevalent in bacterial but not vertebrate genomic DNA (198). However, it has been found in the sera of patients with SLE, and can induce some of the immunological abnormalities that are seen in the disease, such as activating mononuclear cells and inducing the expression of IFN-α, suggesting that it has an important role in the pathogenesis of the disease (199, 200). The immunostimulatory effects of unmethylated DNA are enhanced when it is complexed with anti-dsDNA antibodies (181). A subsequent study found that these DNA containing immune complexes can stimulate several cells that act as APCs, including B cells, PDCs and monocytes (183). Therefore, anti-dsDNA antibodies, when complexed with unmethylated DNA, can act as early mediators of immune dysregulation, which might explain why the first symptoms of the disease typically occur at least one year after the antibodies are first detected in the serum (107).

Anti-ENA antibodies

The role that antibodies to RNA-binding proteins (also known as extractable nuclear antigen (ENA)) have in the disease is less well understood. Immune complexes containing ENA antibodies are also found in the tissue of patients (201), but the presence of these antibodies in the sera do not correlate with active disease (202). Furthermore, whilst some symptoms, such as sicca symptoms and Raynaud's phenomenon are consistently associated with certain ENA antibodies, most clinical manifestations have not been found to have a strong association with any specific ENA antibody (140).

Immunization with $SmD1^{83-119}$, a peptide of the SmD1 ribonucleoprotein, has been shown to lead to the generation of $SmD1^{83-119}$-reactive T cells, which help B cells to produce anti-dsDNA and then anti-$SmD1^{83-119}$ antibodies (203). RNA-containing immune complexes can then activate PDCs by causing the internalization of RNA, which activates TLR-7, to produce pro-inflammatory cytokines (184, 204). They might also be able to activate B cells via BCR/TLR-7 engagement (205). It could therefore be proposed that ENA antibodies are initially produced following antigenic stimulation and then amplify the disease process by stimulating other autoantibody producing B cells and other cells that might be important at later stages in the inflammatory pathway.

Antiphospholipid antibodies

Antiphospholipid antibodies are a heterogeneous group of antibodies that bind to anionic phospholipids (206). They have been associated with a range of manifestations, including recurrent venous or arterial thromboses, foetal loss, eclampsia, thrombocytopaenia, valvular heart disease, small renal artery vasculopathies, livedo reticularis and neurological manifestations, such as transverse myelopathy (207). They have also been associated with small vessel vasculitides, such as Henoch-Schönlein purpura (208). The dominant antiphospholipid antibodies target the proteins prothrombin and β_2GPI, both of which are important in the control of the coagulation pathway. These antibodies have been shown to promote coagulation, but it has also been suggested that they can induce inflammation by

activating the complement pathway, upregulating adhesion molecules by endothelial cells and inducing the expression of cytokines by endothelial cells, monocytes and DCs (206, 209, 210).

Rheumatoid Factor

Rheumatoid factors are a class of autoantibodies that bind to the Fc portion of IgG antibodies. They are present in healthy individuals but class switched rheumatoid factor is associated with systemic autoimmune diseases, including rheumatoid arthritis, primary Sjögren's syndrome and SLE (211). Their role in SLE is unclear but in an animal model they have been shown to increase the internalization of DNA-containing immune complexes into B cells, and activation of B cells via the BCR and TLR signaling pathways (212).

Anti-C1q antibodies

C1q is a collagen-like molecule that binds to and clears apoptotic bodies and antibodies bound to antigen. In SLE antibodies directed agains C1q have been observed in approximately a third of patients. It is seen most commonly in patients with lupus nephritis, particularly proliferative lupus nephritis. It has also been associated with other forms of renal disease. Like anti-dsDNA antibodies they have been found in the glomeruli, where they can induce immune-complex mediated inflammation. They might also influence immunological tolerance to nuclear antigen by dysregulating the normal clearance of apoptotic cells (179).

Cell specific antibodies

In addition to the antibodies to nuclear antigen patients with SLE are also highly prone to developing autoantibodies that bind to proteins on the cell surface, in particular glycoproteins on the surface of red blood cells and platelets (178), or bind to proteins expressed in specific tissue that are associated with an increased predisposition to developing organ-specific autoimmune disease, such as hypothyroidism (213). The large variety of autoantibodies that have been found illustrates the general breakdown in immune tolerance in SLE.

Do autoantibodies cause disease heterogeneity?

Seropositivity for one or more of the antibodies described above is one of the typical features of the disease, but it has been difficult to explain how a limited number of autoantibodies can account for the various different disease manifestations. Studies have tried to correlate these antibodies with the symptoms but whilst some of the antibodies do correlate with symptoms (140), none of them are 100% specific, with the possible exception of antibodies to red blood cells and platelets that are seen in haemolytic anaemia and thrombocytopaenia respectively. Although, antinuclear antibodies are seen in the tissue it is not understood why antibodies to a ubiquitous antigen should deposit in different tissue in each patient and over different periods of time. This has led to studies, which have tried to find whether there are additional antibodies that are more specific to tissue antigen. As a result over 100 autoantibodies have been identified (178), suggesting that the disease might be induced by polyclonal B cell activation. Other studies have examined whether there are differences in the binding of antibody to the antigen in the tissue (214).

Production of pathogenic autoantibodies

Autoantibodies are produced naturally in healthy individuals and are seen as important components of the early immune response to infection. They might also remove products of cell death and inhibit inflammation. However, in autoimmune disease it is suggested that these autoantibodies become more pathogenic as a result of increased affinity of the antibody for the antigen and class switching from the IgM to the IgG isotype (215). Several explanations have been provided for the antibody changes, including molecular mimicry by microbial antigens, protein modification, exposure to proteins that are not normally detectable by leukocytes (e.g. nuclear antigen) by failure of dying cells to be cleared appropriately and defects in the processes that regulate antibody production (216). Some of these processes are primary phenomenon, as a result of genetic polymorphisms or mutations, whilst others are secondary phenomenon due to external factors, such as infection, drugs and ultraviolet light.

Sequential appearance of autoantibodies

It has been shown that several years prior to diagnosis, 88% of patients with SLE have at least one typical SLE antibody that is detectable in the serum (107). The mean interval between detection of the antibody in the serum and the onset of first ACR symptom and then diagnosis was greatest for anti-Ro antibodies (2.97 and 3.68 years respectively) and lowest for anti-nRNP (0.20 and 0.88 years respectively). For anti-dsDNA antibodies the mean interval from first detection in the serum to onset of first ACR symptom and diagnosis was 1.24 and 2.24 years respectively. The antibodies steadily accumulated from a mean of 1.47 out of seven antibodies at six years before diagnosis to 3.01 at the time of diagnosis but the accrual of antibodies halted after diagnosis. Similar observations have been seen in other autoimmune diseases.

These results emphasize that autoantibodies are important in the pathogenesis of disease, but also show that there is not a clear temporal relationship between the appearance of autoantibodies and the onset of symptoms, suggesting that another pathological step or 'hit' is required. However, the authors suggest that there is a "crescendo of autoimmunity culminating in clinical illness" based on the fact that there is a steady accumulation of autoantibodies with different specificities, which has also been seen in another study (217), and supported by a study that showed rising concentrations of autoantibodies before diagnosis (218). The authors also conclude that there are four phases of disease. First there is a normal phase when there are no autoantibodies present and patients are asymptomatic. In the second phase, termed benign autoimmunity, autoantibodies are present without any symptoms. In the third phase, termed pathogenic autoimmunity, autoantibodies that are more specific for SLE, namely anti-dsDNA and anti-Sm appear. Finally, in the fourth phase patients develop clinical symptoms and illness. However, the studies of patients with ILE show that not all patients develop multi-systemic disease even when they have some of the pathogenic antibodies. Therefore, there is possibly an additional phase that accounts for the transition from the onset

of symptoms in just one organ to the development of symptoms in multiple organs (i.e. systemic disease that fulfills the ACR criteria).

The period of benign autoimmunity is poorly understood. The mechanisms that induce the immune system to enter the clinical phase need to be determined to help us predict the onset of clinical disease. Examining changes in the immune system and correlating with changes in symptoms following therapy might shed light on these mechanisms.

1.4.4 The role of toll-like receptors in SLE

TLRs act as an important bridge between the innate and the acquired immune systems. The ligands are highly conserved molecular motifs consistently found in or on the cell surfaces of a range of different pathogens, called pathogen-associated molecular patterns (219). By binding to TLRs, of which there are known to be 10 in humans, they have been shown to induce a number of different functions depending on the cell-type and the TLR ligand involved. These include increasing antigen presentation (220), modulating the cytokine and chemokine secretion profiles (49, 50, 221) and inducing leukocyte differentiation and proliferation (222). By doing this, the immune system can be adapted to respond to infectious organisms or neoplastic cells. However, systemic autoimmune disease appears to be driven in part by inappropriate stimulation of TLRs. In the case of SLE, the TLRs 3, 7, 8 and 9 have been implicated as being important factors in the disease (223, 224). The reason for this association is because the ligands for these receptors are RNA, which bind to the TLRs-3, 7 and 8, and hypomethylated DNA, which binds to TLR-9, and are also the autoantigens recognized by the autoantibodies in SLE. TLRs-7 and 9 are predominantly expressed in PDCs and B cells. TLR-8 is mainly expressed in monocytes (225). TLR-3 on the other hand is not highly expressed by peripheral blood mononuclear cells, but is expressed in renal mesangial cells. Stimulation of these cells with RNA homologues, in mice predisposed to an SLE-like illness, leads to the production of cytokines and chemokines capable of exacerbating the disease (223). These ligands can therefore activate an inflammatory response by stimulating both peripheral blood mononuclear cells (PBMC) and interstitial cells.

Stimulation of TLR-9 in B cells has been shown to induce a number of different responses, including cytokine production, proliferation, upregulation of costimulatory markers and T cell independent antibody production (226, 227). TLR-7 stimulation of B cells has been shown to induce similar responses to those seen following TLR-9 stimulation (228). However, despite the similar effects of TLR-7 and TLR-9 stimulation these receptors do not induce identical immune responses. In fact, one study showed that TLR-9 deficiency but not TLR-7 deficiency in mice, lead to different outcomes in the mice with an unexpected worsening of disease in the TLR-9 deficient animal model for SLE (229). In addition, varying combinations of the TLR ligands can induce different immune responses by DCs when stimulated (230). Further signals are also provided by Fc receptor activation by immune complexes (183). Therefore, the precise effect that the multiple combinations of RNA and DNA antigen complexed with different subclasses of antibodies have on varying levels of B cells and APCs and their respective receptors remains unknown but they could possibly explain the heterogeneous nature of the disease.

Positive feedback of autoantibodies

It is possible that during this period of benign autoimmunity DNA and RNA-containing ICs cause further activation of T cells and/or B cells. The first mechanism, as mentioned above, is by the stimulation of DCs via TLR-7 and/or 9 to prime naïve T cells and produce B cell activating cytokines, in particular IFN-α and BAFF. Secondly, hypomethylated DNA and RNA are potent B-cell mitogens and have been shown in mice to directly activate B cells, in particular to induce secretion of anti-dsDNA and ENA antibodies (231). DNA containing immune complexes can activate human B cells (183) and, therefore, could theoretically also increase the production of autoantibodies. Thirdly, autoantibodies can induce cell death in the tissue, which might lead to the release of more nuclear antigen that can stimulate B cells and other leukocytes.

1.4.5 Cellular abnormalities in SLE

Cellular dysfunction in SLE can be divided into three categories. Firstly, the development of autoantibodies requires a breakdown in B cell tolerance, with possible help from other cells, notably $CD4^+$ T cells (203) and natural killer T (NKT) cells (232). Secondly, leukocytes are seen in the tissue of patients and their presence correlates with functional impairment suggesting a direct role in inducing tissue damage (155). Thirdly, dysfunctional regulatory cells have been described, which may influence the loss of tolerance and/or the immune response within the peripheral tissue (233).

Phenotypic and functional abnormalities have been described in several leukocytes, both effector and regulatory cells, in the blood of patients with SLE. These abnormalities can correlate with disease activity (234-236), autoantibody levels (236-238) and disease manifestations (239, 240). However, correlations with cellular abnormalities do not necessarily imply a causal relationship and might be secondary to the inflammatory process and/or autoantibody stimulation of APCs. The challenge of studying leukocytes in SLE is trying to determine whether the specific cellular abnormalities that are seen in the blood cause humoral dysfunction, tissue or lymph node infiltration, or induce another disease process, and if they are primary or secondary phenomenon.

Autoantibodies can induce cellular dysfunction in SLE

Human *in vitro* studies have demonstrated that autoantibodies, when complexed with nuclear antigen, can stimulate PDCs to produce high levels of IFN-α (181, 182, 184, 241). An important study showed that the IFN-α in the serum of patients with SLE leads to the maturation and activation of monocytes (241). It has since been shown to also increase agrin signaling in T cells (242), whilst IFN-α producing APCs from patients with SLE inhibit the function of regulatory T cells (243) and activate $CD8^+$ T cells (234). IFN-α also has effects on other cell types, such as B cells (244).

A number of other pro-inflammatory cytokines and chemokines can be produced by PDCs stimulated by DNA containing immune complexes from patients with SLE, including IL-1β, IL-8, IL-12, IFN-γ and tumour necrosis factor alpha (TNF-α) (183). Interestingly, B cells and monocytes can also be stimulated to produce IL-8, a chemokine that recruits neutrophils and in mice myeloid DCs can also be stimulated by immune complexes bound to nuclear antigen to produce BAFF and TNF-α (185).

The cytokines IL-1β, IL-12 and TNF-α have been shown to have important effects on T cell differentiation, in particular towards the pro-inflammatory T_H1 and T_H17 subsets (245, 246). Therefore many of the abnormalities seen in the leukocytes of patients with SLE might be a secondary phenomenon due to the effects of autoantibodies stimulating the production of pro-inflammatory cytokines.

As antibodies can dysregulate the cellular arm of the immune system it is difficult to establish which cells are initially responsible for the loss of tolerance to autoantigens and stimulating the production of autoantibodies, particularly in human disease. There is evidence of increased effector memory B and T cells (247, 248) and impaired regulatory cells (249-251). The disease might develop as a result of an imbalance between effector and regulatory cells leading to loss of self-tolerance and the activation of B cells resulting in the production of pathogenic autoantibodies. This hypothesis is supported by several experimental models (203, 252-254) and a study in humans that showed a negative correlation between autoantibody levels and regulatory T cells (255). However, as autoantibodies are present in the blood many years before the clinical features of SLE emerge (107) it is difficult to establish whether the primary event that leads to the loss of self-tolerance occurs first in the B cells, effector T cells or in the regulatory cells. One group has proposed a model that suggests that B cell tolerance is lost first leading to the subsequent dysregulation and accumulation of other leukocytes, supported by a number of studies involving animal models of the disease (256).

B cells

The presence of autoantibodies in patients with SLE indicates a central role for autoreactive B cells in the disease. Natural autoantibodies are considered to be important to immune homeostasis, but these antibodies are normally of the IgM class and have low affinity for antigen (215). Autoreactive B cells that have high affinity to autoantigen are often anergized or deleted (257). In humans there appears to be at least three checkpoints; two during early B cell development in the bone marrow and periphery (258) and a third during the transition from naïve to IgM^+ memory B cell (259). It has been suggested that receptor editing might also be an important mechanism of inducing B cell tolerance (260).

Precisely where the tolerance checkpoints fail in SLE is unclear. There have been only a few studies of human anti-dsDNA antibodies and whilst there have been studies of peripheral B cell tolerance in autoimmune mouse models they might not be applicable to human disease. One study of adolescent patients with SLE showed a high fraction of naïve antigen-inexperienced B cells that are capable of secreting autoantibody suggesting a failure of tolerance during early B cell development (261). Another study showed that three clonally unrelated, somatically mutated anti-dsDNA antibodies from a single SLE patient were due to a failure in at least 2 different tolerance checkpoints and were derived from both self-reactive and non self-reactive B cells (262). Impairment of B cell tolerance in multiple checkpoints (both early and late) is more common in patients with active disease (263). A study of $9G4^+$ B cells that produce autoantibodies that react to self glycoproteins and are increased in SLE showed that these cells fail to be excluded from the germinal centre, possibly due to an anergic response to BCR stimulation (264). Additional factors that might contribute to the checkpoint failures include IL-2, IL-10, BAFF and co-stimulation via CD40L, ICOS and CD70 (237, 265-267). It has been shown that a subset of B cells that produce anti-dsDNA antibodies do not express RP105 (268), which negatively regulates TLR-4 signaling (269). However, studies have not shown if increased TLR-4 signaling has an effect on B cell function.

Disturbances have been reported in both the immature and mature B cell compartment of patients with SLE. There is an increased percentage of immature transitional 1 B cells (270), and in the mature B cell compartment there are reduced frequencies of non-switched memory B cells but increased frequencies of plasma cells and an atypical population of memory B cells that do not express IgD or CD27 but express CD95, particularly during active disease (238, 271, 272). Autoreactive clones that produce autoantibodies have been identified in the switched and non-switched memory B cell population. A population of $CD19^{hi}$ B cells has also been identified which appears to produce antibodies to Sm antigen (238, 271, 273). The role of $IgD^{-}CD27^{-}$ B cells in disease is not known. One study has shown that these cells have gene rearrangement indicating that they are memory B cells that have encountered antigen (238). They also correlated with the presence of pathogenic antibodies, but their ability to produce antibodies or their autoreactivity has not been examined.

B cells appear to have multiple signaling defects (274). These defects either cause upregulation of a signaling pathway that causes differentiation, antibody secretion and proliferation (e.g. PI-3 kinase), cause downregulation of a signaling pathway that causes cell cycle arrest and apoptosis (e.g. ATR) or cause upregulation of a pathway that induces cell migration (e.g. ATR). Some patients with SLE have genetic polymorphisms in the BCR and FcγRIIb signaling pathways (275, 276), suggesting that the defects are intrinsic.

In addition to antibody production B cells are also known to have other functions, including antigen presentation and cytokine secretion (277). An animal model of SLE has shown that the antibody-independent functions of B cells can induce mild disease, although it is less severe than when autoantibodies are present. The precise mechanism by which B cells induce disease without secreting antibodies was not shown but the disease was characterized by the activation and migration of T cells into the renal parenchyma (195). In human studies, B cells from patients with SLE have been shown to produce high levels of cytokines, such as IL-6 and IL-10, which can stimulate B cells in an autocrine manner to produce higher amounts of

antibodies (278-280). IL-6 and IL-10 can also influence non-B cells but it has not been shown if these effects are important in SLE.

There are a number of *in vivo* studies using animal models that suggest that B cells play a direct role in T cell homeostasis (45, 46, 281) and primary B cell defects might therefore be the cause of the abnormalities seen in the T cells of patients with SLE. Regulatory T cells, for example, appear to be expanded by primary resting B cells via TGF-β secretion and antigen presentation but B cell activation results in the death of regulatory T cells (282). Activated B cells have also been shown to induce $CD4^+$ T cell anergy and apoptosis in a contact dependent mechanism (283). B cells might also play an important role in expanding the effector memory T cell pool via CD70 (284), although if this process is uncontrolled there can be continuous conversion of naïve T cells into effector memory cells, depleting naïve T cells from the lymph nodes and spleen, which can result in severe immunodeficiency (285).

The differentiation of B cells into plasma cells is often considered to be a central mechanism of disease, supported by a study that showed that the numbers and frequency of plasma cells positively correlate with active disease and the titre of anti-dsDNA antibodies (128). However, plasma cells can be either short or long-lived. Evidence from human studies suggests that plasma cells that produce antibodies to ENA are usually long-lived but antibodies to dsDNA are usually short-lived (286). Plasma cells are more likely to be long-lived if they arise from a germinal centre rather than an extra-follicular locus and also if they can find a survival niche in the spleen or bone marrow. Interestingly, the plasma cells that correlate with high anti-dsDNA antibody titres have been identified to be HLA-DR^{hi} plasmablasts (287), supporting the possibility that they are arising as a result of autoreactive B cells prematurely exiting the normal tolerance checkpoints. Another study showed that B cells produce anti-dsDNA antibodies after co-stimulation by ICOS on T cells (288). There is evidence that the interaction between B cells and $ICOS^+$ T cells occurs in the kidney (265), suggesting that short-lived plasma cells are produced in non-lymphoid tissue.

There is also evidence to suggest that B cells induce disease via antibody independent mechanisms (195). These mechanisms have not been fully explored, but studies in animals and more recent studies from patients that have B cell depletion therapy show that phenotypic and functional changes occur in $CD4^+$ T cells (289-294). Changes have also been observed in the natural killer (NK) and NKT cell populations (295). These studies suggest that B cells modulate other cells of the immune system either directly or indirectly.

T cells

T cells from the blood of patients with SLE appear activated (296, 297) (237), terminally differentiated (247) and express high levels of pro-inflammatory cytokines (236). They also exhibit a number of signaling defects (298). However, the precise effect that these abnormalities have on the T cells and how each of these abnormalities contribute to the disease remains unclear. Whilst some of the effects of T cell dysfunction in SLE have been shown to contribute to autoantibody production, more recently it has been suggested that T cells might also induce damage directly within the tissue (234). Similarly, although polymorphisms have been identified in the genes that control T cell function of patients with SLE suggesting that they have primary defects, it has also been shown that some of the abnormalities identified are secondary phenomenon (242), possibly as a result of the antibody-induced dysregulation of APCs.

An important role for $CD4^+$ T cells in the pathogenesis of SLE has been supported by the use of anti-CD4 therapy in a small open label clinical trial with five patients with cutaneous disease (299). Clinical improvements were seen in these patients, but a randomized controlled trial (RCT) has not been performed to confirm the result and larger RCTs selectively blocking T cell costimulatory molecules have also failed to confirm a clear role for T cells in SLE (147).

$CD4^+$ T cells are phenotypically and functionally heterogeneous in SLE

Whilst T cells have been shown to have a number of different physiological functions within the immune system, most functional studies in SLE have focused on their ability to help B cells and increase autoantibody production, particularly the pathogenic anti-dsDNA antibodies. In mice predisposed to lupus-like disease memory T cells specific to an Sm peptide have been shown to be critical for increasing anti-dsDNA antibody levels when the mice were injected with an Sm peptide (300). *In vitro* studies showed that the T cells that increased anti-dsDNA antibody production secreted both IFN-γ and IL-4 (203). In human disease T cells specific to an Sm peptide have also been identified and shown to express high levels of TNF-α and IFN-γ but low IL-10, a regulatory cytokine. Disease activity and anti-dsDNA levels positively correlated with the TNF-α secreting memory T cells but negatively correlated with IL-10 secreting memory T cells suggesting that a balance toward pro-inflammatory cytokines induced autoantibody production (236). $CD4^+$ T cells that are specific to histone peptides and RNP have also been identified. Most of these T cells secrete IFN-γ (a T_H1 cytokine) but some also secrete IL-4 (T_H2) and IL-10 (T_R1) (301, 302). The hyperexpression of CD40 ligand by $CD4^+$ T cells might also enhance the production of anti-dsDNA and antinucleosome antibodies (237).

T_{FH} cells are a subset of T cells that reside in the germinal centres and have been shown to induce the differentiation of B cells into long-lived plasma cells (35). In animal models of SLE they induce the synthesis of anti-dsDNA antibodies (303). Circulating levels of T_{FH} cells are elevated in a subset of patients with severe SLE and they correlate with anti-dsDNA antibody titres (304). However, it is not yet known if they induce the synthesis of anti-dsDNA antibodies and/or long-lived plasma cells in the human disease.

The influence of T cells on autoantibody production is supported by the observation that autoantibody-producing B cells are class-switched and have undergone affinity maturation, which requires T cell help. However, studies of animals that get a lupus-like disease have shown that B cell tolerance can be broken without T cell help (305, 306).

Phenotypic analyses show that the memory T cell compartment is substantially increased in SLE compared to healthy individuals (247, 248) but whilst autoreactive T cells specific to nuclear antigens have been found in the peripheral blood it is not known if they make up a significant proportion of this memory T cell population. Likewise, a study has shown that T cells in the peripheral blood and kidneys are clonally expanded in active disease but the clonotypes that accumulate in the kidney are different to the clonotypes that accumulate in the blood and were highly restricted (307). The T cells that migrate into the kidney also have an effector memory phenotype (308). Therefore, whilst a large proportion of $CD4^+$ T cells are more differentiated and clonally expanded there is a suggestion that a large proportion of T cells, particularly the T cells in the non-lymphoid tissue, are not influencing the production of antinuclear antibodies and might have another role in the disease.

T_H1, T_H2, T_H17 and T_H22 subsets have all been implicated as important mediators of disease (236, 309-312). Each subset has been associated with different disease manifestations. Most studies indicate that T_H1 cells are associated with renal disease, although one study has demonstrated that the renal histology differs depending on the T_H1/T_H2 balance (239, 313). T_H1 cytokine expression has been associated with arthritis, whilst T_H2 cytokine expression has been associated with serositis and neurological disease (314). T_H17 cells have been associated with diffuse proliferative lupus nephritis (315) and elevated intrathecal levels of IL-17 have been observed in patients with neuropsychiatric lupus suggesting that T_H17 cells might also be involved in neurological disease (316). T_H22 cells have been associated with skin disease (235).

It is possible that the balance of T helper cell subsets changes during the course of the disease. An animal study has shown that T_H1 type cytokines precede T_H2 type cytokines (317). However, in human disease T_H1 cytokine levels are low in patients with recent-onset disease (318). Instead, T_H17 cells might important in the early stages of human disease (319).

Some studies have examined the expression of chemokine receptors by T cells in patients with SLE. One study showed that $CCR4^+$ T cells, which are associated with T_H2 cells, were low in the blood but elevated in the renal biopsies of patients with nephritis, suggesting that they preferentially migrate to the kidneys (320). Other studies have shown large numbers of CXCR3 and CCR5 expressing T cells in renal biopsies, although in one study they were mostly seen in the tubulointerstitium (134, 321). The T cells in lesional skin appear to express similar chemokine receptors to the T cells seen in the renal lesions (322-324), but in patients with scarring disease the T cells express higher levels of cutaneous lymphocyte antigen (CLA), a skin homing receptor (322).

The costimulatory molecule, inducible T-cell costimulator (ICOS), which is seen on T_{FH} cells and is associated with helping B cells to differentiate into plasma cells, is expressed on $CD4^+$ T cells in inflamed renal tissue close to B cells and Ig deposits supporting the possibility that they induce antibody production in the non-lymphoid tissue (265). Another possibility is that $CD4^+$ T cells recruit and activate other inflammatory cells, in particular neutrophils, monocytes and $CD8^+$ T cells but this does not appear to have been examined yet. It is nevertheless tempting to consider the possibility that the diverse functions and phenotypes of T cells in SLE explain some of the heterogeneous manifestations of disease.

$CD4^+$ Regulatory T cells

Regulatory T cells suppress effector cells from targeting self and harmless environmental antigen (325) and reduced numbers or function leads to either autoimmune or atopic disease (63, 245, 326). As would be expected, a number of defects in regulatory T cells from patients with SLE have been reported. Firstly, regulatory T cell numbers are low (249) and the $CD4^+CD25^{hi}$ cells that are present have been shown to have reduced FoxP3 expression (250), although FoxP3 is unexpectedly increased in $CD4^+CD25^-$ T cells (327, 328). Secondly, $CD4^+CD25^+$ T cells from patients with active SLE have been shown to suppress $CD4^+CD25^-$ T cells inadequately (250), although it is not certain whether this is due to a defect in the functional ability of regulatory T cells or a defective response to regulatory signals in the

effector T cell population (329-331). Thirdly, unlike effector T cells, regulatory T cells are not seen in the tissue of patients with SLE (332), possibly because regulatory T cells from patients with active disease express reduced levels of CCR4 and have reduced migratory ability (333).

Animal studies show that genetic defects in regulatory T cells can lead to lupus-like disease (334). It is possible that the dysregulation of regulatory T cells in human disease might be due to polymorphisms in the genes controlling regulatory T cell function (116), it has also been shown that the dysregulation is secondary to defects in other cells within the immune system (243).

T cell differentiation

Although the precise mechanisms that influence the differentiation of T cells in SLE are not known, they are likely to require antigen-presentation, co-stimulatory signals and cytokine stimulation being provided by APCs, such as DCs and/or B cells. The polarization of T cells towards a particular helper subset depends on the cytokine and co-stimulatory signals provided, which in turn is dependent on the danger signal provided by the pathogen (335). In the case of SLE T cell differentiation is likely to be influenced by the abnormal processing and clearance of apoptotic and necrotic cells. Whilst the clearance of dying cells normally promotes an anti-inflammatory response, in SLE the nucleic acid and nuclear proteins induce pro-inflammatory signals in APCs and B cells leading to release of cytokines such as IFN-α by PDCs, increased MHC expression and increased co-stimulatory molecules such as CD80 and CD86.

There are a number of possible reasons for dying cells causing a pro-inflammatory response in SLE. The apoptotic process appears to be defective with apoptotic bodies being shown to accumulate in the sites of inflammation due to a number of factors, including C1q deficiency and impaired phagocytosis. As a result of the impaired clearance of apoptotic bodies, the antigens undergo novel post-translational modification resulting in cryptic epitopes that can

be recognized by autoreactive T cells. Post-translational modification of DNA also increases the ligation of toll-like receptors in APCs and B cells. Further danger signals by stress-proteins released by dying cells might also amplify the pro-inflammatory signals by APCs (336). However, more importantly the pro-inflammatory response is amplified by autoantibodies complexing with the autoantigen and activating APCs via Fcγ receptors. This is particularly important in SLE because immune-complexes are soluble and therefore it is possible that they can deliver pro-inflammatory signals to APCs and B cells in germinal centres distal from the original source of cell death. As a result, large numbers of T cells can, in theory, be activated and induced to differentiate in the SLOs, such as the spleen, from where they subsequently enter the blood to migrate to the non-lymphoid tissue.

Monocytes and monocyte derived antigen presenting cells

Myeloid cells provide an important bridge between the innate and adaptive immune systems via phagocytosis, toll-like receptor activation followed by cytokine production and antigen presentation to T cells. Circulating monocyte numbers are low in active disease (337), but they have increased expression of activating high affinity Fc receptors, particularly in patients with severe lupus nephritis (338, 339), and there is an increase in the frequency of cells expressing the co-stimulatory molecule CD40L (340). MDC numbers are also low (341) and the cells are also characterized by high expression of activating Fc receptors, co-stimulatory molecules and the pro-inflammatory cytokine IL-8 (342, 343).

Studies in mice show that MDCs are activated by direct stimulation with nuclear antigen (344) and can be induced to produce BAFF or TNF-α when stimulated with either chromatin or nucleosome-containing immune complexes (185). In humans nuclear antigen complexed with the high mobility group box protein 1 induce the maturation of MDCs and can stimulate macrophages to produce pro-inflammatory cytokines (345). Healthy monocytes can be induced to produce IFN-α when stimulated with RNA transfected using lipofectin (184) and IL-8 when stimulated with DNA-containing immune complexes (183). However, DNA-containing immune complexes cannot induce healthy MDCs to produce IL-8.

It is likely that myeloid cells have roles in both the dysregulation of immune tolerance in SLOs and in mediating or exacerbating inflammation in the non-lymphoid tissue. *In vitro* studies show that monocytes from SLE patients function as potent APCs (241) and healthy monocytes stimulated with SLE serum induce $CD8^+$ T cells to become more cytotoxic, which causes them to generate soluble nuclear autoantigen (234) that can activate more myeloid cells. They also directly promote IgG plasmablast differentiation via BAFF and IL-10 stimulation of B cells and IgA plasmablast differentiation via (a proliferation-inducing ligand) APRIL (346).

Macrophages have been seen in the biopsies of the inflamed tissue and the amount of infiltration correlates with the expression of monocyte chemoattractant protein 1 (MCP-1) (347, 348). It is unclear how they contribute to the inflammatory process. It is likely that they are either directly damaging local cells or they are presenting antigen to T cells that are also present. However, MFG-E8-deficient mice have macrophages that cannot engulf apoptotic cells and develop a systemic autoimmune disease as a result (349), suggesting that there is a defect in the function of the monocytes and macrophages that infiltrate the tissue. Patients with SLE have also been shown to have similar defects of phagocytosis (350-352), which in small numbers of patients might be a result of mutations in the MFG-E8 gene (353).

Plasmacytoid dendritic cells

PDCs are derived from a different precursor to MDCs and can be identified by their expression of the surface marker CD123 and lack of expression of the surface markers CD11c (354). They are considered to be the main source of IFN-α, which has a wide range of effects, including the activation of lymphocytes, APCs, plasma cells and endothelial cells, and therefore have a central role in the disease. PDCs can also influence the polarization of healthy T helper cells, but it is unclear whether they influence the different cytokine profiles of T cells that have been seen in patients with SLE.

The production of IFN-α by PDCs can be induced by both DNA and RNA-containing immune complexes by stimulating TLR-9 and TLR-7 respectively (183, 184). This can be inhibited by hydroxychloroquine, one of the key therapies in SLE (355).

It has been shown that PDCs migrate to inflamed lymph nodes (356), where the release of IFN-α could contribute to the local activation of autoreactive naïve B and T cells. Studies have shown that they are also found in the biopsies of active lesions from the skin and kidneys (357-360). One study showed that they can produce IFN-α in the skin, which inhibits the repair of damaged tissue by disrupting normal angiogenesis (361) and recruits $CXCR3^+$ T cells (that are T_H1 biased) by inducing the synthesis of the chemokine IP-10 in the epidermis (358). PDCs can also produce the molecule granzyme B, which can cause the death of local interstitial cells (360).

Granulocytes

Granulocytes have also been implicated in the pathogenesis of SLE. Neutrophils express higher levels of complement receptor-3 (362) and Fas (363). Immune complexes, complement, cell debris and/or cytokines that are present in the tissue can activate them but they are not seen frequently in inflamed tissue, except in the less common lesions (126, 364). It is, therefore, unlikely that they have a significant role in causing tissue damage. However, there is evidence that they are more apoptotic and that the clearance of apoptotic neutrophils by macrophages is impaired (350, 363), which would lead to increased availability of extracellular nuclear antigen to induce the production of antinuclear antibody. Dying neutrophils have also been shown to capture antigen in in extracellular traps, which externalize autoantigens, including dsDNA, and have been shown to be associated with increased anti-dsDNA antibodies, suggesting another mechanism for the breakdown in tolerance to nuclear antigen (365). In mice, basophils that have been activated by autoreactive IgE, promote T_H2 cell differentiation and enhance the production of autoantibodies that cause glomerulonephritis (366). The numbers of basophils in the circulation are lower in patients

with active disease (367), but it is unclear whether they have a significant role in human disease.

In summary, multiple cells, of both the innate and acquired immune system, are associated with the pathogenesis of disease but it is not clear at what stage they each participate in the inflammatory pathway. Therapeutic targeting of individual cells, for example with rituximab, might allow identification of the sequence of cell involvement that eventually leads to disease.

Leukopaenia in SLE

Another central feature of SLE is that circulating leukocyte numbers are usually low in patients with active disease. Lymphopaenia is most common, seen in between 65 and 80% of adult patients at time of diagnosis (368, 369). It correlates to some extent with disease activity and anti-dsDNA antibody levels and has also been associated with anti-ENA antibodies (368, 370-372). One study has demonstrated that the lymphopaenia in untreated SLE is predominantly due to decreases in the $CD4^+$ subset, but reductions are also seen in the $CD8^+$, $CD16/56^+$ natural killer (NK) (297) and B cell subsets (373). In the cutaneous variant of the disease, discoid lupus erythematosus, $CD4^+$ T cells are not decreased, unlike the $CD8^+$ and NK subsets, which might provide some insight into the different mechanisms of disease in each organ manifestations. Neutropaenia is a well described feature of active disease although it is seen in fewer patients than lymphopaenia (369). DC numbers are low, although disease activity only correlates with reductions in the plasmacytoid subset, which is responsible for the production of IFN-α, and does not correlate with reductions in the myeloid subset (359, 374). Circulating monocyte numbers are also low, but it is not clear if this correlates with disease activity (339).

Given the importance of many of these cells in helping autoantibody production and tissue infiltration, the low number of these cells in the blood provides further confusion as to

precisely what their roles are in the disease. If, for example, $CD4^+$ T cells are low in the body how do they then help B cells to differentiate into plasma cells or class-switch and where do the $CD4^+$ T cells in the tissue come from if they are not in the blood?

Several explanations have been given for the cytopaenias that are seen in SLE. The most common is antibody mediated cytotoxicity (type 2 hypersensitivity), either due to antibodies to DNA cross linking with the lymphocyte membrane (375) or to the presence of antibodies that bind to specific cell surface markers such as CD4 and CD45 (376). Other possible explanations include increased apoptosis via soluble factors present in the serum (377-379), Fc receptor ligation by immune complexes (380), cell activation or loss of survival signals (381), bone marrow suppression (382), impaired lymphocyte development due to IFN-α (383) and cell migration (359, 384). Drug toxicity is another possible explanation although this would not account for the leukopaenia that is seen at presentation. Cell migration from the blood into the tissue would be the most logical explanation as this would account for the disappearance from the blood and appearance in the tissue and explain the similar phenotypic abnormalities that are seen in both compartments (322, 359). This explanation is supported by a study that transient lymphopaenia can be induced in mice by IFN-α enhancing the adhesion of lymphocytes to the endothelium (385). It is difficult to explain how cell death would account for leukopaenia in active disease because there is no clear reason why cells in the blood would be more likely to die than cells in the inflamed tissue.

1.4.6 Lymphocyte migration in SLE

Lymphocytic interactions can occur in either SLOs or in the non-lymphoid tissue. The mechanisms that control B and T cell migration are not clear, but they might explain some of the differences that are seen in patients. T cells have been shown to express a number of different chemokine receptors including CCR4, CCR5 and CXCR3, which are expressed on terminally differentiated memory cells. In the skin $CCR4^+$ T cells are seen (322) whereas in kidneys, T cells have been seen to express a variety of different chemokine receptors

including CCR4, CCR5 and CXCR3 (320, 321, 386). It can be assumed that many of these cells are effector cells, but it is possible that some of these T cells are regulatory T cells, which are also known to express CCR4 (387), although in patients with SLE regulatory T cells express reduced levels of CCR4 (333).

The observation that lymphocyte levels are commonly low during active disease, whilst being found in sites of inflammation suggests that T and B cells could be migrating to these areas from the blood. The migration of cells will depend on them expressing chemokine receptors that respond to the specific ligands and chemokines produced in the target tissue. These ligands are produced in different organs to varying levels. RANTES, the ligand for CCR5, for example, is expressed in high levels in the kidney possibly explaining the high levels of $CCR5^+$ T cells that are seen in the kidneys of patients with SLE (388). Just as cellular debris can activate leukocytes it can also induce a pro-inflammatory response in the tissue parenchyma, to recruit inflammatory cells. CpG-DNA, for example, enhances expression of ICAM-1 on endothelial cells (389), whilst RNA enhances the expression of chemokines (223).

1.4.7 Cytokines and chemokines

SLE is characterized by abnormal levels of various different cytokines and chemokines (278, 390-392). Different cell-types produce different cytokines and chemokines under specific stimulation (393). In SLE cytokine dysfunction can be broadly categorized according to those that are derived from APCs and those derived from T cells. Whilst there is general agreement with regards to the abnormal expression of cytokines derived from APCs, there is less agreement with regards to the abnormal expression of cytokines derived from T cells.

APC-derived cytokines

IFN-α has been strongly linked to SLE based on studies that have shown a strong correlation between active disease and activation of genes linked to the IFN-α receptor pathway, called the interferon signature. It has also been observed that administration of recombinant IFN-α

can induce a lupus-like disease (394). PDCs are the main source of IFN-α (395), although one report has suggested that in patients with SLE neutrophils might also be a source (396). It is produced after stimulation of TLR-7 and/or TLR-9 by nucleic acids and Fc receptors by immunoglobulins that bind to nucleic acid as immune complexes. Its effects in the immune system are diverse, but include increasing antigen presentation (via both class I and class II MHC expression), co-stimulation and activation of the NK cells and lymphocytes in order to enhance the cytotoxic immune response to intracellular pathogens, in particular viruses. Studies have shown that the IFN-α in the serum of patients with SLE can induce the differentiation of monocytes to become potent APCs (241), which activate cytotoxic $CD8^+$ T cells that can increase the amount of soluble nuclear antigen and further exacerbate the immune response as a result (234). IFN-α can also induce the differentiation of B cells to become plasma cells but possibly only when PDCs are present (346, 397). There are several different subtypes of IFN-α, which has complicated attempts to develop therapies to block it. Therapeutic strategies that have been attempted include the development of monoclonal antibodies that can neutralize most, if not all, of the IFN-α subtypes, a vaccine that leads to the production of autoantibodies that can neutralize IFN-α and an antibody that can block the IFN-α receptor. Only phase 1 or 2 studies have been completed so far but the results have shown only moderate clinical benefits (398-400).

BAFF (also known as B Lymphocyte Stimulator (BLyS)) is mainly produced by monocytes and neutrophils. It has three receptors, BAFF-R, B cell maturation antigen (BCMA) and transmembrane activator and calcium modulator and cyclophilin ligand interactor (TACI), which can also bind a protein similar to BAFF, called APRIL. BAFF acts primarily on B cells, although the receptors are also found on T cells. High expression of BAFF in mice leads to B cell hyperplasia, hypergammaglobulinaemia and production of anti-dsDNA antibodies. In patients with SLE, high BAFF levels correlate with high levels of anti-dsDNA antibodies (401) and BAFF secreted by DCs stimulated with serum from SLE patients induce IgG plasmablast differentiation (346). The importance of BAFF in SLE has been confirmed by a

RCT, which showed clinical and serological improvements in patients treated with anti-BAFF therapy (402).

Other cytokines that are elevated in SLE include IL-6 and IL-10, which are produced by monocytes, DCs and B cells. These cytokines also have diverse roles including activation of B cells. IL-6 is a pro-inflammatory cytokine that can induce Th-17 differentiation and plasma cell differentiation. However, IL-10 has mostly regulatory effects, but can also promote B cell differentiation into IgG producing plasmablasts, which could be relevant in SLE (346, 403). It is therefore unclear whether it has a primary causal role in disease or is elevated in response to the inflammation but failing to suppress it (251). Blockade of IL-6 has shown clinical and serological improvements in an open-label phase 1 study involving a small group of patients with mild to moderate SLE, although it was accompanied by side effects such as neutropaenia (404). Blockade of IL-10 has also been shown to be beneficial in a small group of patients (405). However, larger RCTs of IL-6 and IL-10 blockade have not yet been reported.

T cell derived cytokines

The role of T cell derived cytokines in SLE is more controversial. Decreased IL-2 production is the most consistent abnormality that has been observed, but IL-15, which is part of the IL-2 superfamily is increased (406). The precise reason for the reduced production of IL-2 and how this might contribute to clinical disease is unclear. IL-2 is important for maintaining T cell survival, in particular regulatory T cells, which are defective in SLE, although a clear link between these two defects has not been established.

It has been hypothesized that cytokines from T cells provide help for B cells and stimulate autoantibody production. Helper T cells can be divided into four subtypes, which have distinct cytokine profiles; T_H1 (IFN-γ), T_H2 (IL-4), T_H17 (IL-17) and T_{FH} (IL-21). Each of these cytokines and/or their T helper subset has been found to be high in SLE (407), and all have been shown to influence antibody production (408-411), but a definite causal link between these cytokines and disease has not been established. However, the diverse range of

cytokines that have all been associated with the disease might explain the wide variety of manifestations that are seen in the disease.

Chemokines

Several chemokines, including MCP-1, TARC, RANTES and IP-10, which bind to CCR2, CCR4, CCR5 and CXCR3 respectively, have been found to be high in the blood and/or urine of patients and correlate with disease activity (388, 412, 413). B cells and PDCs can produce most of the chemokines that are high in SLE following stimulation with TLR-7 or TLR-9 ligands (50, 414-416) or immune complexes bound to DNA (183), suggesting that they are the main source in the disease. There is less evidence of chemokine production by T cells, although they can produce RANTES and produce higher levels in patients with SLE because of reduced expression of microRNA-125a, an inhibitory post-transcriptional regulator of RANTES expression (417). Endothelial and tissue-derived cells can also produce some of these chemokines, either as a result of antibody stimulation (418), IL-1β stimulation by infiltrating IgG-stimulated lymphocytes (419) or TLR ligation by RNA (223, 420).

1.5.1 Rituximab – mechanism of action

Rituximab is a chimeric mouse/human monoclonal antibody that binds to the cell surface antigen CD20, which is expressed on all B cells from the pre-B cell stage to the mature B cell stage. The precise mechanisms by which B cells die following binding to rituximab are unclear, but are thought to be by antibody-induced cell mediated cytotoxicity, complement-induced lysis and/or induction of apoptosis (421). It was first used in the treatment of B cell lymphomas, being approved by the Federal Drug Administration in November 1997. It has since been used in the treatment of autoimmune diseases including autoimmune thrombocytopaenia, haemolytic anaemia and rheumatoid arthritis. The efficacy of B cell depletion in autoimmune disease has often been attributed to the improvement in autoantibody titres that are thought to contribute to these diseases. However, rituximab has

also been used successfully in autoimmune diseases where a role for autoantibodies is not firmly established leading to the suggestion that B cells might be exerting antibody-independent roles in specific diseases (422, 423).

1.5.2 B cell depletion therapy in SLE

Given that B cells, by producing autoantibodies, have been shown to have such a central role in SLE, in both animal models and human disease, it was hypothesized that the depletion of B cells could be an effective means of treating patients. B cell depletion therapy (BCDT) was first used for the treatment of SLE at University College London Hospitals (UCLH) in 2000 in patients that were refractory to conventional immunosuppressants (424). In this open-label study 6 patients with SLE previously refractory to all other conventional treatments were given 2 infusions of rituximab 2 weeks apart in addition to 2 infusions of cyclophosphamide and 1 infusion of methylprednisolone. One patient was lost to follow-up, but the remaining 5 patients all showed clinical and serological improvements at 6 months and there were no serious adverse events. A retrospective study of rituximab therapy given to 50 patients with SLE was then later reported in order to assess the efficacy further. This study reported complete remission in 42% of patients (defined as change in the BILAG A or B scores to C or D in every organ or system), partial remission in 47% of patients (defined as a change in the BILAG A or B scores to C or D in one or more organs or systems but with a persistent BILAG A or B score in one organ or system) and non-responsiveness to BCDT in 11% of patients that were available for assessment at least six months after the first cycle of therapy. A response to therapy was seen in all organ systems, although the percentage of patients that responded was lowest in the renal system compared to the other organ systems (425). Similar observations were seen in four other small open-label studies (426-429) and a prospective study from a national registry (430). However, two RCTs subsequently failed to confirm that BCDT is effective in SLE and lupus nephritis (431, 432).

A number of possible explanations have been given for the failure of RCTs to demonstrate that BCDT is clinically effective (146). One important reason is that BCDT was compared

against a combination of high dose prednisolone and mycophenolate mofetil, which is known to inhibit activated B cells (433) and is very effective in SLE (434). BCDT was not compared against placebo alone, which would have probably observed that B cell depletion is an effective treatment in SLE, even if it is not necessarily superior to the currently used medications. A second important point is that the RCTs rely on the assumption that B cells exert equal and/or similar effects in all patients. However, B cells have multiple functions, in addition to antibody synthesis, and these functions might vary between patients with SLE. Understanding these differing functions and stratifying patients accordingly might lead to better outcomes in RCTs.

Serological factors that predict clinical response or relapse post BCDT

Reductions in the mean anti-dsDNA antibody and increases in C3 levels paralleled improvements in clinical disease in all but one study. Early relapse is associated with a reduction in anti-dsDNA antibody levels following BCDT (435), the presence of anti-ENA antibodies and hypocomplementaemia at baseline (436) and higher baseline levels of BAFF (437).

As B cells repopulate, there is a variable increase in anti-dsDNA and anti-C1q antibody levels between patients (292) but studies have not shown if relapse coincides with a rise in autoantibody levels or a fall in complement levels. One small study of four patients showed that as B cells repopulated in two patients who subsequently relapsed, there was a rise in the levels of anti-dsDNA antibodies bearing the idiotope recognized by the 9G4 monoclonal antibody (435).

Cellular factors that predict clinical response or relapse post BCDT

Higher numbers of B cells at baseline have been associated with poorer clinical response (427). This association could possibly be explained by the earlier repopulation time of B cells in patients with higher B cell numbers at baseline, which might be due to higher BAFF levels as has been observed in a separate study of patients that relapse early (437).

Three studies have shown that the time to B cell repopulation varies considerably between patients, but did not observe a clear relationship between relapse and the repopulation of B cells. Each study, however, used different definitions of B cell repopulation and disease assessment tools, which might partially explain the differences in the results that were observed.

The first study of 15 patients showed that the median time to repopulation, defined as more than 0.005 x 10^9 B cells/l, was five months (range, 4-11), but excluded two patients who had not repopulated during the study period, of whom one was deplete of B cells for over three years (435). Only six patients relapsed within one year of BCDT and one patient relapsed before B cells had repopulated. However, of the four patients that relapsed within one year of BCDT, four relapsed within one month of B cell repopulation suggesting that there is a close relationship between B cells and relapse in a subset of patients.

The second study of 16 patients showed that the median time to repopulation, defined as more than 0.01 x 10^9 B cells/l, was seven months (range, 2-12), but excluded one patient who had not repopulated four years after therapy (427). B cells had repopulated in all seven patients that relapsed but the median time to relapse was 18 months (range, 10-40).

The third study of 31 patients showed that the median time to repopulation, defined as more than 0.02 x 10^9 B cells/l, was 10 months (range, 2-16), although the definition of B cell repopulation was higher than has been used in other studies. The analysis also excluded 13 patients whose B cells had not repopulated during the study period, of whom one had B cells deplete for 31 months (428). Eighteen patients relapsed with a median time of 11 months (range, 4-24). Of the 18 patients that relapsed only 10 had repopulated, although its definition of B cell repopulation was slightly higher than in the other studies. The median time between B cell repopulation and relapse was 6.5 months (range, 1-26) in those 10 patients.

The reasons for the different definitions of B cell depletion in the three studies were not given. Whilst the differences in the B cell threshold between the studies do not appear

substantial it is noticeable that the study with the higher threshold for B cell depletion had the highest percentage of patients that relapsed without the B cells appearing to have repopulated. Evidence is required to define B cell depletion, i.e. the number of circulating B cells that contributes to clinical disease.

B cells comprise multiple different subsets, and it is likely that relapse is dependent on the repopulation of one or more specific subset rather than all of the B cells. One study has shown that the initial B cells that repopulate are naïve and that remission correlates with a delay in the reconstitution of $CD27^+$ memory B cells (438). A second study also showed that early repopulation of $CD27^+$ memory B cells and high numbers of circulating plasmablasts at week 26 was associated with early relapse (429). Other B cell subsets, such as the IgD^-CD27^- memory B cell, have also been shown to correlate with active disease but the relationship between the repopulation of these cells and relapse following BCDT have not been reported. Identifying all the subsets that are involved in the pathogenesis should improve the use of B cell counts in monitoring disease following BCDT.

B cell depletion has also been shown to be associated with reductions in T cell activation markers, CD154, CD69 and HLA-DR (290, 294, 439) with improvements in regulatory T cell function (293), numbers, defined by the marker CD25 (290, 292), and Foxp3 expression (440). Two studies have also reported increases in the total numbers of $CD4^+$ T cells in the blood (290, 292). Increased numbers of $CD8^+$ T cells, NK cells and monocytes were also seen in one of the studies (292). However, it is not clear whether these changes were a direct result of the inhibition in B-T cell interaction or an indirect effect of reductions in autoantibody levels. In addition it is not clear how these T cell abnormalities specifically affect the disease process. Most studies have focused on the role of T cells in helping B cells make autoantibodies, but this is irrelevant if B cells are not present to differentiate into plasma cells and if the plasma cells are long-lived and continue to secrete autoantibodies independently of other cells, as has been observed.

Histological changes following BCDT

Only one study has examined the changes in the cellular infiltrates of the inflamed tissue following BCDT from patients with SLE. This study examined the changes in the renal biopsies from six patients who were treated for active nephritis. Two of the six biopsies showed a moderate/large leukocytic infiltrate in the tissue after BCDT and three showed occasional leukocytic infiltrates. The infiltrates were mostly in the renal interstitium rather than the glomerulus. Most of the cells that were present were T cells and plasma cells, but in the two samples where there were moderate/large infiltrates a few B cells were also seen. Only one biopsy was taken both before and after B cells repopulated, which, therefore, made it difficult to establish if the B cells that were present in the biopsies of the other 5 patients were there as a result of repopulation and reinfiltration or if they were just not fully depleted. In the paired biopsies that were taken T cell numbers were low and B cells were undectectable at depletion, having both been present in higher numbers before BCDT, and both then increased after repopulation indicating that the cells had re-infiltrated the tissue. Interestingly, this was the only patient in the study that had low anti-dsDNA antibody levels (441).

In a recent study of patients with multiple sclerosis who were treated with BCDT it was demonstrated that lower numbers of T cells were present in the cerebrospinal fluid of treated patients. These changes correlated with reductions in the chemokine CXCL13. Interestingly there were no significant changes in IgG levels or oligoclonal bands suggesting that the clinical response was due to the inhibition of T cell trafficking (442).

1.6.1 Model of disease

There are four key disease stages in SLE. The first stage is the defective clearance of antigen from dying cells causing the activation of APCs. The second stage is the breakdown in T and B cell tolerance that leads to the production of autoantibodies, in particular antinuclear antibodies. The third stage is DC activation by immune complexes bound to nuclear antigen causing further dysregulation of B and T cells. In the final stage autoantibodies bind to tissue antigens causing a complement cascade, leukocyte recruitment or thrombosis. The proposed sequence of events is shown in Figure 1.6.1, similar to models proposed by others (443, 444).

However, a number of observations do not fit this proposed model. Firstly, whilst over 100 autoantibodies have been identified (178), very few are present in more than 25% of patients and correlate well with disease manifestations (140) or activity (127). They are often present even when there are no signs or symptoms of clinical disease (107). Secondly, therapeutic interventions, such as BCDT, do not always show a clear correlation between the treatment response and the clearance of circulating autoantibodies (33, 435). Thirdly, lymphocytic infiltrates are seen in the tissue (126), but it is not clear if these cells infiltrate the tissue before or after antibodies deposit in the tissue. The precise role of these cells and how they fit into the disease stages needs clarifying.

To achieve more meaningful results from clinical trials a much clearer understanding of the disease is required, one that would allow the sequence of events that lead to symptoms and organ damage to be predicted. This thesis will try to answer two critical questions, which I believe will improve our understanding of the disease and help us to plan future clinical trials.

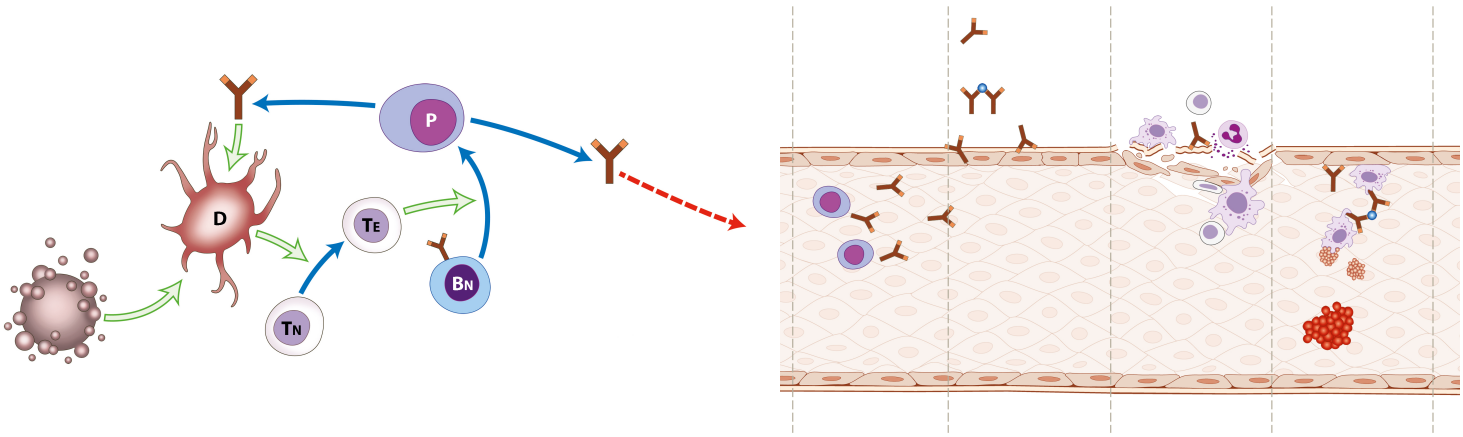


Figure 1.6.1 – A proposed sequence of events leading to inflammation and damage in the tissue.
In the early stages nuclear antigen released by dying cells activates myeloid dendritic cells (D), which then present antigen and activate autoreactive T cells. Naïve autoreactive T cells (T_N) then differentiate into effector T cells (T_E), which provide help to naïve autoreactive B cells (B_N) to differentiate into autoantibody secreting plasma cells (P). This stage starts in the circulation and therefore precedes the disease symptoms. In the later stages autoantibodies then either enter the tissue from the circulation and bind to antigen where they induce a complement cascade, bind to endothelial cells and recruit inflammatory cells, such as lymphocytes, neutrophils and macrophages to cause vasculitis or induce platelet, red blood cell and/or leukocyte aggregation within the blood vessel to cause thromboses. Vasculitis and thrombosis can be primary or secondary events.

Circulating autoantibodies do not always correlate with active inflammation. One possible explanation is that a second 'hit' is required to cause autoantibodies to enter the tissue, which depend on antibody-independent functions of B cells. This thesis will examine the sequence of events that occur as B cells repopulate after BCDT in order to identify the source of autoantibody production and how they might get into the tissue.

Key: Green arrows represent stimulation, blue arrows represent differentiation, red arrows represent migration or transfer into non-lymphoid tissue.

1.6.2 How do antibodies get into the tissue?

The presence of antibodies and complement in the tissue is another defining aspect of the disease. However, it is not clear how antibodies enter the tissue from the blood. One possibility is that they are produced by plasma cells that are present in the tissue but this would then lead to the question of what mechanisms control the migration of B cells and/or plasma cells into the tissue. The second possibility is that antibodies are produced in SLOs and then cross the vascular endothelium from the blood. However, the latter explanation leads to the question of whether the endothelium is normally permeable to antibodies, actively transports antibodies from the blood or if the endothelium requires signals to allow passive transfer of antibodies. The ability of the vascular endothelium to allow transfer of autoantibodies to cross into the tissue has been examined in autoimmune diseases of the nervous system, which show that the disruption of the endothelial junctions is dependent on T cell interactions with endothelial cells (445-448). Vascular integrity does not appear to have been examined in SLE, although it has been suggested that it is likely to be an important factor in cerebral lupus (449). Arguably, vascular integrity is not as important in glomerulonephritis, as the glomerulus is more permeable to proteins than the endothelium around the brain.

Assuming that the transfer of antibodies into the non-lymphoid tissue is an active process then it is likely to be independent of the process that regulates the production of autoantibodies, i.e. it is a second step or 'hit'. This process needs to be clarified to understand fully the sequence of events that lead to clinical disease and, therefore, the kinetics of the disease. It might also help us to understand how some therapies might control the inflammatory process.

1.6.3 Can B cells induce disease via an antibody independent mechanism?

To date there has only been one study examining the possibility of an antibody-independent role for B cells in SLE (195). This was carried out in an MRL/*lpr* mouse strain, in which the B cells were genetically modified to not be able to secrete antibody. It was reported that the

mice that had B cells that could not secrete antibodies (mIgM Tg) had a higher mortality than mice without B cells. Intriguingly, whilst glomerular pathology was almost absent in the mIgM Tg mice, interstitial nephritis and vasculitis persisted, dominated by the presence of large numbers of perivascular T cells. In addition evidence of T cell activation could be seen in the splenic T cells suggesting that activation was occurring in the SLOs, most likely as a result of direct interactions between the B and T cells.

The use of the MRL/*lpr* mouse strain has been criticized because it is heavily dependent on a single gene accelerator and they develop profound lymphoproliferation, which is not characteristic of the human disease (450). However, it is one of the few animal models that has been shown to develop multi-systemic disease, involving the joints, skin, kidneys and brain as is seen in human SLE. The mice also develop vasculitis. By removing the antibodies from the MRL/*lpr* mouse strain Chan *et al* were able to examine the role of antibody-independent function of B cells in causing inflammation in multiple organs.

The study raised two important points. The first was to suggest that some of the inflammatory lesions that are seen in SLE and contribute to tissue damage and mortality, in particular the perivascular and interstitial lesions, are a result of T cell infiltration, whilst other lesions, such as glomerular lesions are due to immune complexes. The second was to show that this T cell infiltration was dependent on the presence of B cells. The external mechanisms that control T cell migration are not fully understood and whilst B cells have been shown to have multiple functions that are not related to antibody synthesis, such as antigen presentation and cytokine secretion, there have been no studies that have examined how B cells might influence T cell migration into the non-lymphoid tissue in SLE. Studies have also not examined the antibody independent roles of B cells in human autoimmune disease. However, a number of studies indicate that several cellular changes occur following BCDT that might not be due to changes in antibody levels (290).

1.7.1 Summary

SLE is a heterogeneous and unpredictable disease. It is defined by the presence of antinuclear antibodies in the serum and immune complexes in non-lymphoid tissue, although the target tissue can vary between patients. Current understanding of the pathogenesis is that apoptotic antigen stimulates PDCs to make cytokines, which activate T cells and then B cell differentiation into autoantibody-producing plasma cells (Figure 1.6.1). However, there is a lack of evidence of a clear causal and temporal relationship between circulating antibodies and the inflammation in the tissue, with the possible exception of anti-dsDNA antibodies and glomerulonephritis, suggesting that an additional process or 'hit' is also required for disease.

There are two specific questions regarding how antibodies/ immune complexes deposit in the tissue that have not been clearly explained; firstly, where are the autoantibody secreting plasma cells formed and if they originate in SLOs how do the antibodies that they secrete cross the vascular endothelium? B and T cells interact with each other and they both have functions that are not directly related to the production of antibodies. These functions might explain where antibodies are made and how they get into the tissue. The kinetics of this process might explain the period of 'benign autoimmunity'. By examining the interactions between B and T cells and by appreciating both their antibody-dependent and independent functions, it should also be possible to get a better understanding of the disease and the differences in the response to therapies, including BCDT.

2 HYPOTHESIS

2.1.1 Hypothesis

During the period of benign autoimmunity, one or many active events occur that eventually lead to inflammation in the tissue, which is often characterized by the presence of immune complexes and complement. An additional 'hit' that either controls the production of antibodies inside the tissue or the dysregulation of the vascular endothelium is probably required. I hypothesize that that this additional process is mediated by an antibody-independent function of B cells.

Rituximab therapy offers an opportunity to examine the serological and cellular changes that occur in the circulation and tissue as B cells are first depleted and then repopulate and would help to identify the sequence of events that lead to the production of pathogenic autoantibodies and/or the dysregulation of the vascular endothelium.

2.1.2 Aims of this study

1. To establish the sequence of events that lead from remission ('benign autoimmunity') to disease by correlating the immunological events that occur as B cells repopulate following rituximab therapy with new symptoms and clinical events.
2. To identify factors that influence B cell repopulation following rituximab therapy.
3. To determine whether B cells interact with other cells in the immune system, in particular $CD4^+$ T cells, and where in the body these interactions occur.

3 METHODS

3.1.1 Samples

Blood samples were obtained from patients who attended the clinic for autoimmune rheumatic disease at University College London Hospital (UCLH). Patients were defined as having SLE if they fulfilled the ACR revised classification criteria for SLE (101). Healthy control samples were obtained from colleagues at the Windeyer Institute for Medical Research and medical students from University College London. Blood was obtained by consent with approval from the local ethics committee.

40mls of blood was collected into a 50ml plastic Falcon tube containing 100µl of heparin, 4mls of blood was collected into a BD vacutainer serum tube containing a clot activator and gel for separation and which was coated with silicone and another 4mls of blood was collected into a BD vacutainer whole blood tube containing 7.2mg K_2 ethylenediaminetetraacetic acid (EDTA).

3.1.2 Assessment of disease activity

Disease activity of the patients with SLE was assessed using the British Isles Lupus Assessment Group (BILAG) index. This is a composite scoring system developed to assess disease activity in patients with SLE who invariably have heterogeneous symptoms and mutliple organ involvement. It has also been developed to discriminate between an active on-going disease process and longstanding damage in the specific affected organs. It was established to offer more than just a global score assessment of disease activity in SLE and was based on the priniciple of the physician's 'intention to treat'. Various studies have shown that the BILAG index has good inter-rater and within-rater reliability and valid when compared to the 'gold standard' criterion, which is the intention to treat, with an overall sensitivity of 87% and specificity of 99% (451). There is also a high positive predictive value overall of 80%, and for each-organ based system, with the exception of the neurological system. It has been compared to other scoring systems, such as the SLAM and SLEDAI, and

has been found to be the most sensitive instrument for measuring fluctuations in disease activity following the commencement of specific therapy (452, 453). It is therefore the best instrument for measuring individual responses to BCDT.

The clinical features, which make up the majority of the assessment, were recorded independently by the physicians in the rheumatology clinic. Patients attended on average every 2 months. They were asked in detail about their symptoms, had a physical examination including a blood pressure measurement and a urine analysis for protein levels and had blood tests. These tests included a full blood count with white blood cell differential, urea, creatinine and electrolyte levels, liver function tests, an erythrocyte sedimentation rate, complement-3 (C3) and anti-dsDNA antibody levels. If indicated, blood tests are also carried out for muscle enzymes, a direct Coombs' test, anti-cardiolipin antibodies and circulating anticoagulant, amongst others. Additional tests such as a chest x-ray, pulmonary function, nerve conduction, urine microscopy, muscle, lung or kidney biopsy and magnetic resonance scans were also requested if clinically indicated. The clinical data for each individual organ-based system are recorded on a BILAG assessment form (appendix 1). The results of the relatively few blood tests required for the BILAG assessment were added later by the clinic nurse. Eight organ-based systems are included on the form; general, mucocutaneous, nervous system, musculoskeletal, cardiorespiratory, vascular, renal and haematology. The data were entered by the clinic nurse onto a computer, which determines the BILAG scores/grades. A grade is thus assigned to each organ-based system depending on the results of the clinical assessment; **A** denotes disease considered sufficiently active to require high dose steroids (intravenous methylprednisolone/ oral prednisolone > 20mg daily) or an increase in the immunosuppressant medication, **B** denotes less active disease with mild symptoms that can be corrected with symptomatic therapy such as anti-malarials, non-steroidal anti-inflammatory drugs or a low dose of steroids, **C** denotes stable mild disease, **D** denotes system previously affected but currently inactive and **E** denotes system never involved. A numerical score can be allocated to each category; **A** equals 9, **B** is 3, **C** is 1 and both **D** and **E**

are 0. A global score is calculated by adding all of the scores given to each organ-system. Active disease is considered to be denoted by a BILAG score of greater than 6. Flare was defined as any new grade A or two new Bs.

Although they do not affect the BILAG score, tests were also requested for erythrocyte sedimentation rate, C3 levels (measured by laser nephelometry) (normal = 0.9 to 1.8 ng/ml) and dsDNA antibody levels (measured by ELISA; Shield Diagnostics Dundee, UK) (normal < 50 Units/ml) as these are also indicative of active disease. However, these tests are not useful for all patients as many patients have active disease despite having normal levels of these markers (132) and vice versa (454). Antibodies to extractable nuclear antigens are assessed once every two years approximately, but exact titres are not calculated as these typically do not change or correlate with disease activity.

3.1.3 B cell depletion therapy

Between June 2000 and September 2010 79 patients were treated with BCDT in the department for rheumatology at UCLH. Patients were referred for BCDT by the clinicians at UCLH either if they had not responded to conventional immunosuppressants (azathioprine, mycophenolate mofetil, methotrexate, cyclosporin and/or cyclophosphamide) or developed unwanted side effects (75 patients) or if they presented *de novo* to the rheumatology clinic without previous use of immunosuppressants (4 patients).

BCDT consisted of an infusion of 100-250mg methylprednisolone followed by an infusion of 1 gram of rituximab, repeated after 14 days. Most patients were also given an infusion of 500mg to 750mg cyclophosphamide the day after the infusion of rituximab, or in the case of the newly diagnosed patients, started on additional oral immunosuppression with azathioprine. Patients continued on lowering doses of prednisolone and hydroxychloroquine (400mg once a day) if they were taking them prior to BCDT.

The circulating numbers of total lymphocytes, B cells, T cells and monocytes were measured at the haematology laboratory at UCLH. Complement levels were measured in the

biochemistry laboratory and autoantibody levels were measured in the immunology laboratory.

3.2.1 Separation of PBMCs

PBMCs were extracted by layering the blood gently onto Ficoll Hypaque at a dilution of 2:1 into a 50 ml Falcon tube. The tubes containing the blood were then centrifuged at 850rpm for 35 minutes at 20°C. The resulting leukocyte layer was then extracted using a Pasteur pipette, transferred to another Falcon tube and then made up to 50mls with RPMI. They were then centrifuged again at 500rpm for 12 minutes at 8°C. The supernatant was poured out and the pellet was gently resuspended in 10mls of RPMI. The cells were counted and 5 x 10^5 PBMCs would then be plated into 300μl U-shaped wells in 96 well plates for *ex vivo* analyses. The remaining cells were centrifuged again at 500rpm for 12 minutes at 8°C. The supernatant was poured away and the resulting pellet was resuspended in freezing medium (foetal calf serum (FCS) with 10% dimethyl sulphoxide (DMSO)) at a concentration of 1 x 10^7/ml in 1 ml cryovials (Nunc™). The cryovials were stored at –80°C. When the cells were required for use they were thawed in a 37°C waterbath and washed twice in 10 mls of 1 x phosphate buffered saline (PBS).

3.2.2 Serum extraction

Blood obtained in the serum tube was left to stand for 30 minutes. The tube was then centrifuged for 10 minutes at 1000 rpm. The supernatant was then extracted and stored in 2 ml cryovials at –80°C.

3.2.3 Depletion and isolation of B cells from PBMCs

B cells were depleted from the PBMCs prior to culture. PBMCs were first washed in 1 x PBS and then divided into two equal aliquots. One aliquot was then spun down and resuspended in 100 to 200 μl of 1xPBS and labelled with antibodies that bound to CD19 attached to magnetic MicroBeads (Miltenyi Biotec, Cat no. 130-050-301) by incubating the cells with the

antibodies for 20 minutes at 4°C. They were then washed twice in 2 mls of 1xPBS and then resuspended in 1 ml of 1 x PBS. An LD column (Miltenyi Biotec) that had been pre-cooled to –20°C was then suspended on a MidiMACS separator (Miltenyi Biotec). The separator contained a magnet to attract the cells bound to magnetic MicroBeads and allows non-bound cells to continue to flow through the column. The LD column was prepared by gently pipetting 1 ml of 1 x PBS onto the column. The solution was collected in a plastic falcon tube. Once this solution had passed through the column the 1ml cell suspension was then also gently pipetted onto the column. Again the suspension was collected in the plastic falcon tube. After the suspension was collected in the tube a further 2 mls of 1 x PBS were added to the LD column, 1 ml at a time to wash any remaining unlabelled cells out of the column. Once all of the cells had been collected the cell suspensions were washed twice in 5mls of complete medium and then resuspended in complete medium at a concentration of 1.5 x 10^6 cells/ml.

In separate experiments, B cells were isolated from PBMCs by negative selection using a B cell isolation kit containing a cocktail of biotin-conjugated antibodies against CD2, CD14, CD16, CD36, CD43, CD235a (Glycophorin A), i.e. surface markers that are not expressed by B cells, and anti-biotin MicroBeads (Miltenyi Biotec, Cat No. 130-091-151). The cells were first labeled with the antibodies by incubating them together for 5 minutes at 4°C and then incubated with the anti-biotin MicroBeads for an additional 10 minutes at 4°C. The cells were then suspended in 500 ml of 1 x PBS and then passed through an LS column using the same method described above.

3.2.4 Counting of PBMCs for *in vitro* studies

PBMCs for *in vitro* studies were counted using a haemocytometer. 10 µl of the cell suspension was diluted in trypan blue solution in a 1:2 dilution. 10 µl of the cell/trypan blue suspension was then counted on a haemocytometer, which contained a 5x5 grid. Dead cells were detected by increased intracellular staining with trypan blue. Live cells were counted

within the grid using a microscope and multiplied by the dilution of the suspension in trypan blue and then by the dilution in medium/1 x PBS to determine the total number of cells obtained.

3.2.5 Cell culture and reagents

3 x 10^5 PBMCs were cultured in 200 μl complete medium (containing RPMI 1640 with 10% FCS and 1% penicillin and streptomycin) in 300 μl U-shaped wells in 96 well plates. The cells were stimulated with either CpG ODN 2006 (Autogen Bioclear; Cat. no. tlrl-hodnb), goat $F(ab')_2$ anti-human IgM, (Southern Biotech, Cat. No. 2022-14) or goat $F(ab')_2$ anti-human Ig (IgM, IgG and IgA) (Southern Biotech, Cat. No. 2012-01) with goat $F(ab')_2$ anti-human IgD (Southern Biotech, Cat. No. 2032-01). Endotoxin-free plasmid DNA (pcDNA3.1; Invitrogen) was provided by Jane Samuel (post-doctoral researcher at UCL). B and T cell interactions were inhibited using functional grade purified human anti-CD2 antibody (ebioscience, Cat. No. 16-0029-85).

Once the cells had been cultured for an adequate period of time the supernatants were gently extracted and stored at –20°C until required for chemokine analysis or the cells were stained for flow cytometric analysis.

3.2.6 Flow cytometry

Cells were stained for surface markers for flow cytometric analysis in 96 well plates. First they were washed three times with 200 μl of FACS buffer (1xPBS with 0.1% sodium azide and 1% FCS). They were then incubated with colour-conjugated antibodies for the markers in Table 3.2.1 and refrigerated in the dark at 4 to 8°C for 30 minutes. They were then washed three more times in FACS buffer to remove excess antibody before being fixed in 100 μl of 2% parafomaldehyde. The cells were then diluted in sheath buffer and the expression of the cell markers were recorded on a BD FACSCalibur or BD LSR platform using Cellquest software. The data were analysed using FlowJo software.

Immunogen	Colour 1	Colour 2	Colour 3
CD19	PE (ebioscience)	PE-Cy7 (ebioscience)	APC (ebioscience)
IgD	FITC (BD)		
CD27	PE (BD)		
CD4	FITC (ebioscience)	APC (BD)	
CD69	PE (BD)	PE-Cy7 (BD)	
HLA-DR	FITC (BD)	PE (BD)	
CD49d	APC (BD)		
CD45RA	PE (BD)	PE-Cy5 (BD)	
CD62L	FITC (BD)		
CXCR3	PE-Cy5 (BD)		
CCR4	PE-Cy7		
CCR5	FITC (BD)		
CCR7	PE-Cy7 (BD)		

Table 3.2.1 – Antibody reagents for flow cytometry.
Abbreviations – FITC **(**fluorescein isothiocyanate**)**, PE (phycoerythrin), PE-Cy5 (phycoerythrin-cyanine 5), PE-Cy7 (phycoerythrin-cyanine 7), APC (allophycocyanin)

3.2.7 Gating

The gating of lymphocytes is illustrated in Figure 3.2.1. B cells were subsequently identified by the high expression of the surface marker CD19. T cells were identified by the high expression of the surface marker CD4.

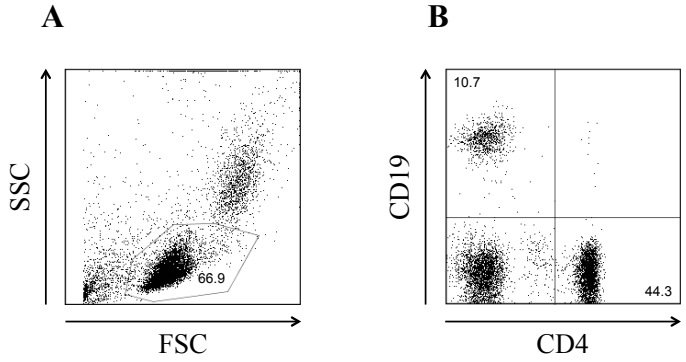


Figure 3.2.1 – Gating of lymphocytes
Representative flow cytometric dot-plots showing the gating of live lymphocytes in the PBMCs of a patient with SLE prior to BCDT (**A**) and the CD4 and CD19 subsets within the live lymphocyte gate (**B**). Values shown are the percentage of cells within the gate.

3.2.8 Cytometric Bead Array

Chemokine levels in the serum and supernatants were measured by cytometric bead array (CBA) using a Human Chemokine Kit (BD, Cat. No. 552990) according to the manufacturers protocol. First a vial of lyophilized Human Chemokine Standards was reconstituted with 4 ml of assay diluent, gently mixed and left at room temperature for 15 minutes to equilibrate. The assay diluent with reconstituted protein was then serially diluted nine times. One tube containing only the assay diluent was prepared to serve as the negative control. The chemokine capture beads were then mixed by adding an aliquot (10 μl per test) from each vial into a single tube and vortexed thoroughly. 50 μl of the capture bead mixture was added to each assay tube with 50 μl of the assay diluent (negative control), standard dilutions or the sample being tested, followed by 50 μl the chemokine PE detection reagent. The assay tubes were incubated for 3 hours at room temperature in a dark cupboard and wrapped in aluminium foil to protect it from light. Afterwards 1 ml of wash buffer was added to each

assay tube and the tubes were then centrifuged at 200 g for 5 minutes. The supernatant from each assay tube was then aspirated and discarded. The bead pellets were resuspended in 300 ml of wash buffer. The samples were acquired on a BD FACSArray and then analysed using FCAP Array software.

3.3.1 Statistical analyses

Statistical analyses were carried out using GraphPad Prism 4 Version 4.0c software for results chapter 5.1 and GraphPad Prism 6 Version 6.0b software for results chapters 5.2, 5.5, 5.6 and 5.7. A p value of < 0.05 was considered significant.

Chapter 5.1

Wilcoxon signed-rank test was used to compare paired data before and after BCDT. Mann–Whitney test was used to compare unpaired data between groups. Kaplan–Meier curves were generated to compare time to B-cell repopulation. Log rank test was used to examine differences between the curves. Associations between the changes in anti-dsDNA antibody levels and duration of B cell depletion were tested by Pearson's correlation coefficient (r_p) for linear relationships. Lines of best fit were estimated by regression analysis. Fisher's exact test was used to determine the association of renal disease and raised anti-dsDNA antibody levels.

Chapter 5.2

Wilcoxon signed-rank test was used to compare paired data before and after BCDT. Mann–Whitney test was used to compare unpaired data between groups. Associations between B and T cell subsets were tested by Pearson's correlation coefficient (r_p) for linear relationships and Spearman's rank correlation coefficient (r_s) for non-linear relationships. Lines of best fit were estimated by regression analysis. Changes in B cell numbers after BCDT were estimated using non-linear regression and the differences between groups were tested by repeated measure ANOVA using the extra sum-of-squares F test. Kaplan–Meier curves were generated

to compare time to clinical relapse. Log rank test for trend was used to examine differences between the curves.

Chapter 5.5

Wilcoxon signed-rank test or paired t-test was used to compare paired data before and after BCDT and before and after stimulation of PBMCs *in vitro* and Mann–Whitney test was used to compare unpaired data between groups. Associations between HLA-DR expression by $CD4^+$ T cells and $CD4^+$ T cell numbers were tested by Spearman's rank correlation coefficient (r_s) for non-linear relationships.

Chapter 5.6

Two-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test was used to compare the means between the groups. One-way ANOVA was used to compare trends between groups. Mann-Whitney test was used to compare groups with data from healthy controls. Kaplan-Meier estimates were used to determine the median time to clinical relapse following BCDT.

Chapter 5.7

Associations between anti-dsDNA antibody and chemokine levels were tested by Pearson's correlation coefficient (r_p) for linear relationships. Two-way ANOVA followed by Sidak's multiple comparisons test was used to compare data between groups of patients treated with BCDT. Paired t-test was used to compare paired data before and after BCDT. Wilcoxon signed-rank test was used to compare paired data before and after stimulation of PBMCs and B cells *in vitro* and Mann–Whitney test was used to compare unpaired data between groups.

All data that was obtained from patients that attended the research phlebotomy clinic were included in the analyses in this thesis. Missing data were either a result of patients being lost to follow-up or non-attendance at the research phlebotomy clinic.

4 PATIENTS

4.1.1 Patients

Data were obtained from 71 of the 79 patients who were treated with BCDT in the department of adult rheumatology at UCLH and eight patients who were treated in other departments at UCLH or other hospitals in London or Birmingham between June 2000 and September 2010. Clinical data for these patients are shown in Table 4.1.1 and summarized in Table 4.1.2. One patient died four months after BCDT from myocarditis, approximately one month after her B cells returned (patient 23). Three patients died at least 18 months after the first cycle of BCDT (one from peritonitis (patient 5) and two from suicide (patients 38 and 70)). Eight of the 79 patients who were treated with BCDT in the department of adult rheumatology at UCLH were not included in the study because of the following reasons; there was insufficient data available and they were lost to follow-up prior to the commencement of my PhD (five patients), they were followed-up in other departments (two patients) and one patient died two weeks after BCDT was initiated because of a reaction to cyclophosphamide.

ID	Ethnicity	Gender	Organ involvement	Previous treatment	Age at diagnosis	Age at BCDT	Cyc with BCDT*
2	W	F	arthritis, serositis, skin vasculitis	pred, hcq, mtx, cya	28	35	yes
3	W	F	arthritis, serositis, lymphadenopathy	pred, hcq, mtx, aza, cya	26	36	yes
5	AC	F	arthritis, serositis, nephritis(IV), skin vasculitis	pred, hcq, aza, cyc, mmf	13	18	yes
6	W	F	arthritis, nephritis(IV), rash	pred, hcq, aza, cyc	9	20	yes
9	A	M	arthritis, serositis, nephritis (IV)	pred, aza, cyc	15	26	no
10	AC	F	arthritis, nephritis (IV), rash	pred, hcq, aza, cyc, mmf	15	30	yes
11	O	F	fever, rash, serositis, nephritis (IV), neuropsychiatric	pred, aza, cyc, cya, mmf	11	27	yes
12	AC	F	arthritis, nephritis (IV)	pred, hcq, cyc	31	38	no
13	W	F	fever, arthritis, nephritis (IV)	pred, hcq, aza, cyc	15	18	yes
14	W	F	TCP	pred, hcq, aza	20	26	yes
15	W	F	fever, arthritis, CNS vasculitis, nephritis (IV)	pred, aza, cyc, mmf	15	19	yes
16	AC	M	haemolytic anaemia, nephritis (IV)	pred, aza, cyc	16	23	yes
17	W/O	F	nephritis (IV)	pred, aza, cyc, mmf	20	21	yes
19	W	F	fever, arthritis, nephritis (IV), rash	pred, hcq, aza, cyc, mmf	20	31	yes
21	AC	F	arthritis	pred, hcq, mtx, aza, cyc,	37	40	yes
22	W	F	arthritis, nephritis (IV)	pred, hcq, aza, cyc	26	31	yes
23	AC	F	fever, arthritis, nephritis (IV), organic brain syndrome	pred, hcq, aza, cyc	23	24	yes
24	A	F	fever, arthritis, serositis, skin vasculitis	pred, hcq, mtx, aza, cyc	16	35	yes
25	A	F	fever, rash, arthritis, anaemia	pred, hcq, mtx, aza, cyc	19	33	yes
26	W	F	arthritis, nephritis (IV), gut vasculitis	pred, cyc, mepacrine	17	36	yes
27	O	F	rash, nephritis (IV)	pred, hcq, mmf	29	33	yes
28	W	F	photosensitivity, arthritis, serositis	pred, hcq, mtx, aza, cyc	17	42	no
29	A	F	mouth ulcers, alopecia, arthritis, serositis	pred, hcq, mtx, ssz, TNF	34	35	yes
30	W	F	arthritis, serositis, myositis, nephritis (IV)	pred, hcq, mtx, aza, ssz, IVIG	23	33	yes
31	W	F	TCP	pred, hcq	27	30	yes
32	AC	F	arthritis, ILD	pred, hcq, mtx, lfn	N/A	49	no
33	W	F	arthritis, pancytopaenia, nephritis (IV)	pred, hcq, aza, mmf	16	17	yes
34	W	F	arthritis, nephritis (IV)	pred, hcq, mtx, aza	13	22	yes
35	A	F	arthritis, nephritis (IV)	pred, aza, mmf, cyc	18	21	yes
36	O	F	arthritis	pred, ssz, aza, mtx	20	23	no
38	A	F	arthritis, neuropsychiatric, nephritis (II)	pred, mmf, mepacrine	36	39	yes
39	AC	F	arthritis, fatigue	pred, hcq, mtx,	31	57	no
40	W	F	skin vasculitis, TCP	pred, hcq	17	41	no
41	A	F	arthritis, rash, serositis	pred, hcq, mtx, cya	19	21	yes
42	A	F	arthritis, nephritis (IV)	pred, aza, mmf	43	51	yes
43	A	F	discoid rash, scarring alopecia, APS	pred, hcq, mtx	20	36	yes
44	O	F	APS (PE, DVT), nephritis (IV)	pred, mmf, cyc	22	47	N/A
45	A	F	neuropsychiatric	pred, hcq, aza, cyc, mmf	23	33	yes
46	W	F	arthritis, serositis, nephritis	pred, hcq, mmf, cyc	43	49	N/A
47	A	F	arthritis, discoid rash	pred, hcq, aza	14	27	N/A
48	A	F	arthritis, rash, neuropsychiatric (epilepsy)	pred, hcq, aza,	27	29	yes
49	AC	F	arthritis, rash, nephritis	pred, aza	37	50	yes
51	W	F	rash, nephritis, gut vasculitis, pulmonary haemorrhage	pred	41	53	yes

ID	Ethnicity	Gender	Organ involvement	Previous treatment	Age at diagnosis	Age at BCDT	Cyc with BCDT*
52	W	F	rash, arthritis, myositis	pred, mtx	33	49	no
53	A	F	arthritis, rash, alopecia, serositis, haemolytic anaemia	hcq, aza, pred	14	24	yes
54	AC/A	F	arthritis, rash, nephritis	pred, aza, cyc	18	46	yes
55	A	F	arthritis, gut vasculitis	pred, ssz, mtx	27	27	no
56	W	M	nephritis, mouth ulcers, rash, alopecia	pred, cyc, mmf	35	40	yes
57	AC	F	arthritis, rash, neuropsychiatric, TCP	pred, aza, cyc	28	33	yes
58	W	F	rash, arthritis	pred, aza, mtx, mmf	50	57	no
59	W	F	arthritis, rash, nephritis (III, V)	pred, aza, mepacrine, mmf	30	49	yes
60	W	M	rash, arthritis	pred, aza, mtx	23	40	yes
61	A	F	arthritis, alopecia, haemolytic anaemia	pred	10	23	yes
62	AC	F	arthritis, rash	none	47	48	yes
63	AC	F	arthritis, serositis, rash	hcq, pred, aza	38	47	yes
64	W	M	rash, arthritis, nephritis	pred, cyc, mmf	12	25	yes
65	O	F	arthritis, alopecia	hcq	27	27	no
66	A	F	arthritis, rash, nephritis (IV)		24	28	yes
67	AC	F	arthritis, nephritis	pred, aza	24	28	yes
68	AC	F	arthritis, discoid rash, alopecia, nephritis (V), serositis	pred, mtx	18	22	no
69	W	F	arthritis, serositis, rash, mouth ulcers	pred, aza	34	45	yes
70	O	F	arthritis, rash	none	21	21	no
71	AC	F	arthritis, serositis, mouth ulcers, nephritis	pred, hcq, aza, mtx	21	24	yes
72	W	F	arthritis, rash	pred, hcq, aza, mtx	44	55	yes
73	AC/W	F	fever, arthritis, rash	pred, hcq, aza, cyc	26	28	yes
74	A	F	nephritis, arthritis, fever	pred	25	25	yes
75	W	M	arthritis	pred, mtx	66	73	yes
76	A	F	arthritis, alopecia, mouth ulcers, nephritis (III + V), skin vasculitis	pred, aza	29	50	N/A
77	AC	F	rash, arthritis, alopecia, PE (APS negative), lymphadenopathy	hcq, pred, aza	8	21	N/A
78	A	F	arthritis, rash, serositis, myositis, skin vasculitis	pred	14	24	yes
79	W	F	arthritis, serositis		39	39	yes
A	W	F	arthritis, APS, TCP	N/A	59	N/A	N/A
B	W	F	arthritis, rash, TCP, nephritis (IV)	pred, aza, cyc	32	N/A	N/A
C	W	F	rash, arthritis	N/A	18	N/A	N/A
D	A	M	arthritis, rash, ILD, nephritis (V), myositis	N/A	18	18	yes
E	AC	M	rash, nephritis (V)	pred, aza, cyc	24	27	N/A
F	A	F	arthritis, rash, alopecia, serositis, nephritis (II)	hcq, pred	7	14	yes
G	AC	F	rash, alopecia, TCP, arthritis, fever	pred	13	13	yes
H	AC	F	arthritis, rash, nephritis (III, V)	N/A	19	N/A	N/A

Table 4.1.1 – Baseline characteristics of patients with SLE treated with BCDT
The abbreviations are explained on the next page

Abbreviations for Table 4.1.1:

A (South Asian), AC (Afro-Caribbean), O (Oriental/ Far East Asian), W (White/ Caucasian), F (female), M (male), CNS (central nervous system), MRI (magnetic resonance imaging), APS (anti-phospholipid syndrome), DVT (deep vein thrombosis), PE (pulmonary embolus), TCP (thrombocytopaenia), ILD (interstital lung disease, pred (prednisolone/corticosteroid), LN (lymphadenopathy) aza (azathioprine), hcq (hydroxychloroquine), mtx (methotrexate), cyc (cyclophosphamide), mmf (mycophenolate mofetil), ssz (sulphasalazine), cya (cyclosporine), IVIG (intravenous immunoglobulin), lfn (leflunomide), TNF (anti-tumour necrosis factor), N/A (data not available).

Lupus nephritis classification: I = minimal mesangial, II = mesangial proliferative, III = focal proliferative, IV = diffuse proliferative, V = membranous

Patients 2 to 79 were treated with BCDT in the department for rheumatology at UCLH. Patients A to H were treated with BCDT in the department for haematology at UCLH (patient A), the department for adolescent rheumatology at UCLH (patients D, F and G) or in other hospitals within London (patients B, C and E) or Birmingham (patient H). Some baseline and follow-up data from patients A to H are therefore not known.

*Refers to whether cyclophosphamide was given with rituximab during the patient's first cycle of BCDT. Treatment regimens with subsequent cycles of BCDT were sometimes adjusted.

Age at diagnosis: mean (range, number)	24.8 (7 – 66, 78)	
Age at BCDT: mean (range, number)	33.3 (13 -73, 75)	
Gender:	**Number/ 79**	**Percentage**
Female	71	89.9
Male	8	10.1
Ethnicity:	**Number/ 79**	**Percentage**
Caucasian	30	38.0
South Asian	21	26.6
Afro-Caribbean	19	24.1
Far East Asian	6	7.6
Mixed	3	3.8
Organ involvement:	**Number/ 79**	**Percentage**
Arthritis	66	83.5
Renal	39	49.4
Rash	38	48.1
Serositis	19	24.1
Cytopaenia	12	15.2
Alopecia	11	13.9
Neuropsychiatric	6	7.6
Skin vasculitis	6	7.6
Gut vasculitis	3	3.8
CNS vasculitis	1	1.3
Pulmonary haemorrhage	1	1.3
Interstitial lung disease	2	2.5
Myositis	4	5.1
Fever	10	12.7
Antiphospholipid syndrome	3	3.8
Cyclophosphamide with first cycle of BCDT:	**Number/ 79**	**Percentage**
Yes	56	70.9
No	13	16.5
Data not available	10	12.7

Table 4.1.2 – Summary of baseline characteristics of patients with SLE treated with BCDT

5 RESULTS

5.1 B-cell numbers and phenotype at clinical relapse following BCDT differ according to anti-dsDNA antibody levels.

5.1.1 Introduction

It was hypothesized that if B cells have an important role in SLE then early relapse following BCDT would be associated with faster rates of B cell repopulation. It was also hypothesized that differences in the time to relapse and the number of B cells and B cell phenotype associated with relapse would vary depending on anti-dsDNA antibody levels. Therefore, the first aim of this study was to examine changes in B cell numbers in patients with SLE following BCDT.

5.1.2 Results

A more rapid rate of B-cell repopulation in patients who relapse earlier

The median B-cell number before treatment was 0.122×10^9/l (range $0.010–0.679 \times 10^9$/l). Following BCDT all patients achieved B-cell depletion, defined as a B-cell count of $<0.01 \times 10^9$/l. A Kaplan-Meier analysis showed that the median time to repopulation (defined as a B-cell count of $>0.01 \times 10^9$ B cells/l) following BCDT was 32 weeks and to clinical relapse (defined as a new BILAG A score or two new Bs) was 66 weeks. The rate of repopulation was greater than the rate of relapse ($p < 0.001$, log-rank test) (Figure 5.1.1A).

To examine whether early repopulation was associated with early relapse, patients were divided into three groups according to whether they repopulated before 16 weeks ($n = 10$), between weeks 16 and 32 ($n = 22$), or after 32 weeks ($n = 29$). A Kaplan–Meier analysis showed that early repopulation was associated with early relapse ($p = 0.0002$, log-rank test) (Figure 5.1.1B). Furthermore, 39 and 45% of patients had inactive disease for >2 years in the group of patients that repopulated between 16 and 32 weeks, and after 32 weeks, respectively, compared with only 10% of patients who repopulated before 16 weeks.

Higher B-cell numbers can be seen as early as 8 weeks following BCDT in those patients who relapsed early (defined as occurring before 18 months, $n = 37$) compared with patients who relapsed late (>18 months, or remain in remission, $n = 24$) ($p = 0.0008$, Mann-Whitney U test) (Figure 5.1.1C).

B-cell numbers at relapse differ according to anti-dsDNA antibody levels

B-cell numbers at relapse were lower than the B-cell numbers at baseline ($p = 0.006$, Mann-Whitney U test) (Figure 5.1.2A). Two distinct patient groups were identified based on anti-dsDNA antibody levels; one with high levels (>100 IU/l, $n = 17$) where relapse occurred with low B-cell numbers and a second with low or normal levels (<100 IU/l, $n = 20$) where relapse occurred with normal or high B-cell numbers (Figure 5.1.2B). Thus patients with levels of anti-dsDNA antibodies >100 IU/l relapsed with lower B-cell numbers than patients with low or normal levels of anti-dsDNA antibodies ($p = 0.02$, Mann-Whitney U test). However,

patients with high levels of anti-dsDNA antibody levels at baseline do not relapse earlier even though they relapse with fewer B cells. There was no correlation between B-cell numbers and C3 levels (data not shown).

I next analysed whether anti-dsDNA antibody levels modified the association between earlier disease relapse and a faster rate of repopulation as shown in Figure 5.1.1. There is not a clear definition of B cell repopulation in SLE and therefore two values were chosen for this study; a low value of 0.01×10^9 B cells/l, because this was the lowest baseline value of the patients in this study and the median value used in three previous studies of BCDT in SLE (427, 428, 435), and a high value of 0.11×10^9 B cells/l, because this was the lowest value of the healthy controls used in this study (Figure 5.5.1B). Disease relapse before 18 months (early) was significantly associated with earlier repopulation to B cell numbers $>0.01 \times 10^9$ B cells/l in patients with high anti-dsDNA antibody levels ($p < 0.01$, log-rank test) (Figure 5.1.2C), but not low or normal anti-dsDNA antbibody levels (Figure 5.1.2D). However, repopulation to B cell numbers $>0.11 \times 10^9$ B cells/l) did not occur any more rapidly in patients with high anti-dsDNA antibody levels in earlier relapsers (Figure 5.1.2D). In contrast, patients with low or normal anti-dsDNA antibody levels who relapsed before 18 months attained B-cell numbers $>0.11 \times 10^9$ B cells/l more rapidly than patients who relapsed after 18 months with low anti-dsDNA antibody levels ($p = 0.01$, log-rank test) (Figure 5.1.2D).

The difference in the median time to repopulation ($>0.01 \times 10^9$ B cells/l) between those patients with high anti-dsDNA antibody levels who relapsed early and those who relapsed later or remained in remission was 13 weeks (25 weeks in the early relapse group, 38 weeks in the late relapse/remained in remission group). Conversely, in patients with low levels of anti-dsDNA antibodies early relapse was not associated with more rapid repopulation to $>0.01 \times 10^9$ B cells/l. However, this latter group attained higher B-cell numbers ($>0.11 \times 10^9$ B cells/l) earlier than patients who relapsed late or remained in remission. The difference in the median time to repopulation to this higher B-cell level between early and late relapse/remained in remission in patients with low anti-dsDNA levels was >58 weeks [48 weeks (early) compared with >104 weeks (late)].

A decrease in anti-dsDNA antibody levels was associated with remission in patients with high levels at baseline

To determine whether changes in anti-dsDNA antibody levels correlated with clinical relapse following BCDT, levels of anti-dsDNA antibodies were analysed at four time points: baseline, B-cell depletion, B-cell repopulation and relapse/remission (Figure 5.1.3A). Remission was defined as no relapse within 12 months of repopulation. In patients with high levels of anti-dsDNA antibodies who remain in remission ($n = 9$), there was a significant decrease in levels of anti-dsDNA antibodies between baseline and repopulation ($p < 0.01$, Wilcoxon matched-pairs sign rank test). Despite this decrease, anti-dsDNA antibody levels remained significantly higher in these patients compared with those who had low or normal levels at baseline and remained in remission ($n = 15$) ($p < 0.05$, Mann-Whitney U test), but not with those who had low or normal levels at baseline and then relapsed ($n = 20$). Levels of anti-dsDNA antibodies did not fall at any time point in patients with high levels at baseline who relapsed ($n = 17$), rather there was a significant increase in anti-dsDNA antibody levels associated with relapse ($p < 0.05$, Wilcoxon matched-pairs sign rank test). Overall, there was a larger percentage decrease in the levels of anti-dsDNA antibodies in those patients who who had baseline levels >100 IU/l and remain in remission ($n = 8$) compared with those who relapsed ($n = 14$) ($p = 0.01$, Mann-Whitney U test) (Figure 5.1.3B). Correlating the decrease in anti-dsDNA antibody levels with the duration of B-cell depletion indicates that there was a greater decline in anti-dsDNA antibody levels the longer B cells remained $< 0.01 \times 10^9$ B cells/l ($n = 48$) ($r_p = -0.41$, $p = 0.003$) (Figure 5.1.3C).

Different B-cell phenotypes correlate with disease relapse

Given that patients with high anti-dsDNA antibody levels relapsed with lower B-cell numbers than patients with low anti-dsDNA antibody levels, I hypothesized that there might be differences in B-cell subsets according to anti-dsDNA antibody levels in patients during relapse and remission. In a subset of patients ($n = 32$), I analysed the different B-cell subsets in relapsing patients and those who remained in remission. As shown in Figure 5.1.4, patients

who had high anti-dsDNA antibody levels at relapse (n = 6) also had increased percentages of $IgD^{-}CD27^{hi}$ plasmablasts ($p < 0.01$, Mann-Whitney U test) compared with patients who remained in remission (n = 9). In contrast, patients who had low or normal levels of anti-dsDNA antibodies had an increased percentage of $IgD^{-}CD27^{-}$ (double negative) memory B cells at relapse (n = 10) compared with patients who did not experience a disease flare (n = 7) ($p = 0.01$, Mann-Whitney U test).

Cyclophosphamide is not associated with differences in the rate of B cell repopulation or the time to disease relapse

Cyclophosphamide was given to most of the patients with rituximab as part of the BCDT treatment regimen. It is an alkylating agent that can affect bone marrow function (455). It was therefore hypothesized that it might affect the rate of B cell repopulation and/or the time to disease relapse. However, Figure 5.1.5A shows that the rate of B cell repopulation was not significantly different between patients that were treated with cyclophosphamide (n = 45) compared to patients that were not treated with cyclophosphamide (n = 11). Figure 5.1.5B shows that the time to disease relapse was also similar in patients that were treated with cyclophosphamide (n = 53) compared to patients that were not treated with cyclophosphamide (n = 12). These results support studies that showed that cyclophosphamide does not impair B-lymphopoiesis in mice (456), and that rituximab monotherapy is as effective as combination therapy with cyclophosphamide in lupus nephritis (457). It would therefore appear that other factors are influencing the rate of B cell repopulation and the mechanisms that cause disease relapse.

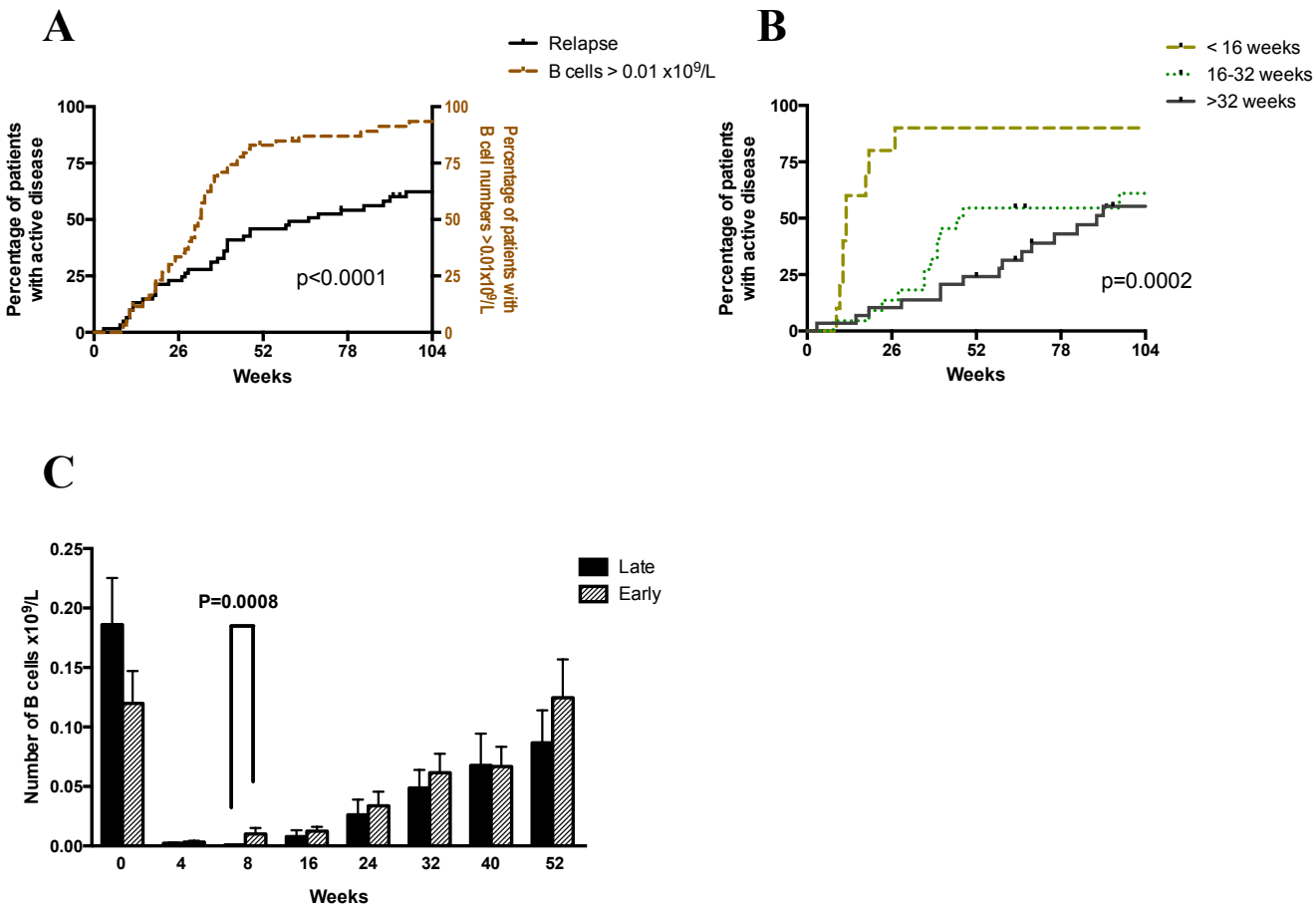


Figure 5.1.1 – The rate of B-cell repopulation compared with the time to clinical relapse in patients with SLE following treatment with rituximab therapy.

(**A**) Kaplan–Meier curves comparing the time to B-cell repopulation [$>0.01 \times 10^9$ B cells/l] with the time to clinical relapse following treatment with rituximab therapy [n = 61] and (**B**) comparing the clinical relapse rates in patients who repopulate before 16 weeks [n = 10], between weeks 16 and 32 [n = 22], and after 32 weeks [n = 29]. Analysis was carried out using the log rank test. (**C**) B-cell numbers are shown from baseline to week 52 between patients who relapse before 18 months [early, n = 37] after treatment and patients who relapse (or remain in remission) after 18 months [late, n = 24]. Columns indicate the mean B-cell number and bars indicate the S.E.M. Differences between groups were analysed by the Mann–Whitney U test.

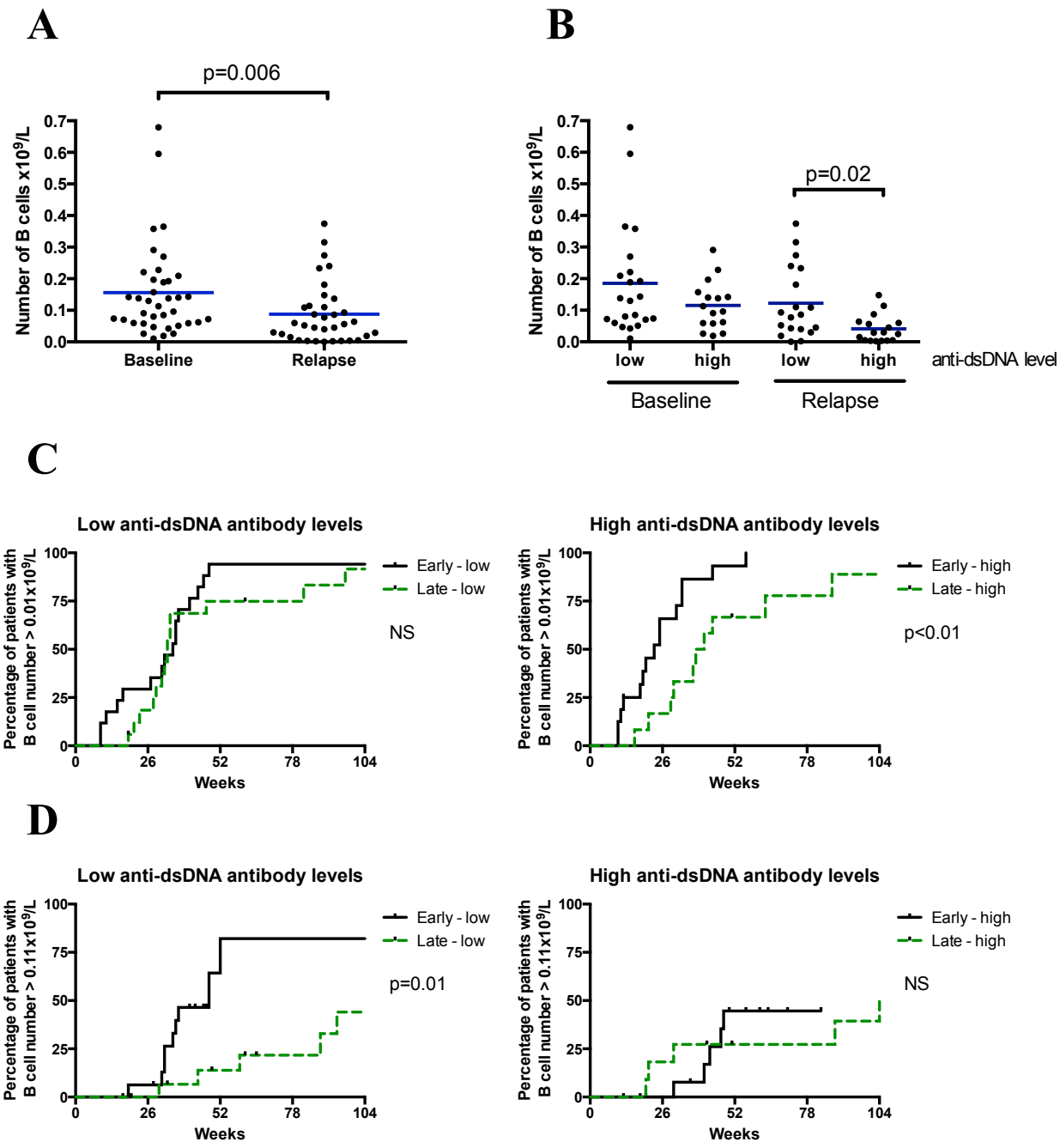


Figure 5.1.2 – B-cell numbers in rituximab-treated patients who relapse with low anti-dsDNA antibody levels compared with those that relapse with high anti-dsDNA antibody levels

(**A**) B-cell numbers at baseline [n = 38] and clinical relapse [n = 36] and (**B**) B-cell numbers at baseline and relapse divided according to whether anti-dsDNA antibody levels were low [low (<100 IU/l); n = 22 (baseline), n = 19 (relapse)] or high [high (>100 IU/l); n = 16 (baseline), n = 17 (relapse)] at relapse. Data are shown as dot-plots with lines representing the mean. Differences between groups were analysed by the Mann–Whitney U test. (**C**) Kaplan–Meier curves comparing time to B-cell repopulation $>0.01 \times 10^9$ B cells/l and (**D**) $>0.11 \times 10^9$ B cells/l in patients who relapse either before (early) or after 18 months (late) with baseline anti-dsDNA antibody levels that were low (<100 IU/l) [n = 17 (early), n = 17 (late)] or high (>100 IU/l) [n = 16 (early), n = 12 (late)]. Comparisons of the curves were carried out using the log-rank test. NS = $p > 0.05$.

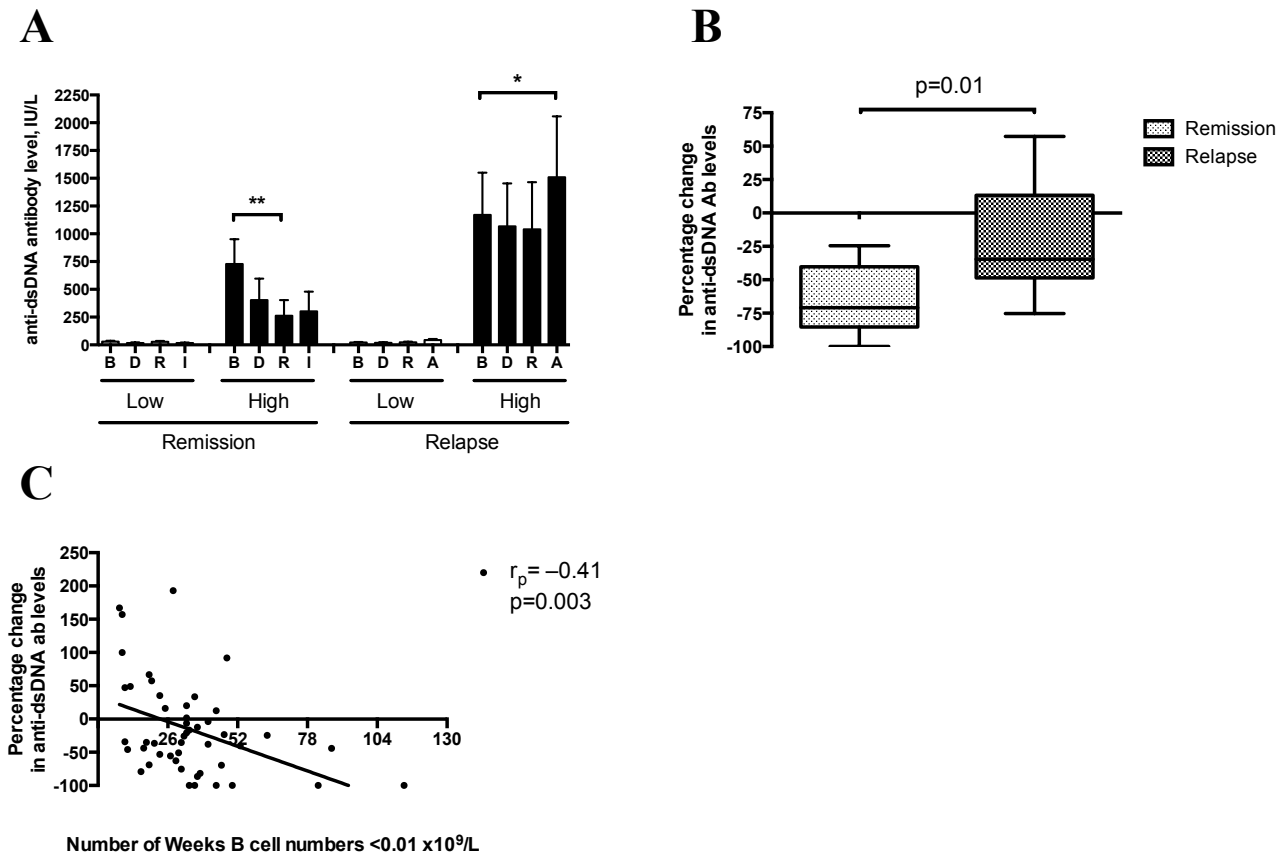


Figure 5.1.3 – Changes in anti-dsDNA antibody levels following BCDT in relapsing patients compared with those that remain in remission

(**A**) Anti-dsDNA antibody levels are shown at baseline (B), during B-cell depletion (D), at B-cell repopulation (R) and at remission (I) or clinical relapse (A) in patients who had anti-dsDNA antibody levels that were low [Low (<100 IU/l); n = 15 (remission), n = 20 (relapse)] or high [High (>100 IU/l); n = 9 (remission), n = 17 (relapse)] at baseline. Columns indicate the mean; bars indicate the S.E.M. Differences between the groups were analysed by the Wilcoxon matched-pairs signed rank test (**B**) Percentage change in anti-dsDNA antibody levels after BCDT in patients with high levels at baseline that go into remission [n = 8] or relapse [n = 14] after B cells repopulate. Lines inside the boxes indicate the median; outer borders of the boxes indicate the 25th and 75th percentiles; bars extending from the boxes indicate the range. Differences between the groups were analysed by the Mann–Whitney U test. (**C**) Correlation between the change in anti-dsDNA antibody levels prior to B-cell repopulation and the time to B-cell repopulation [n = 48].

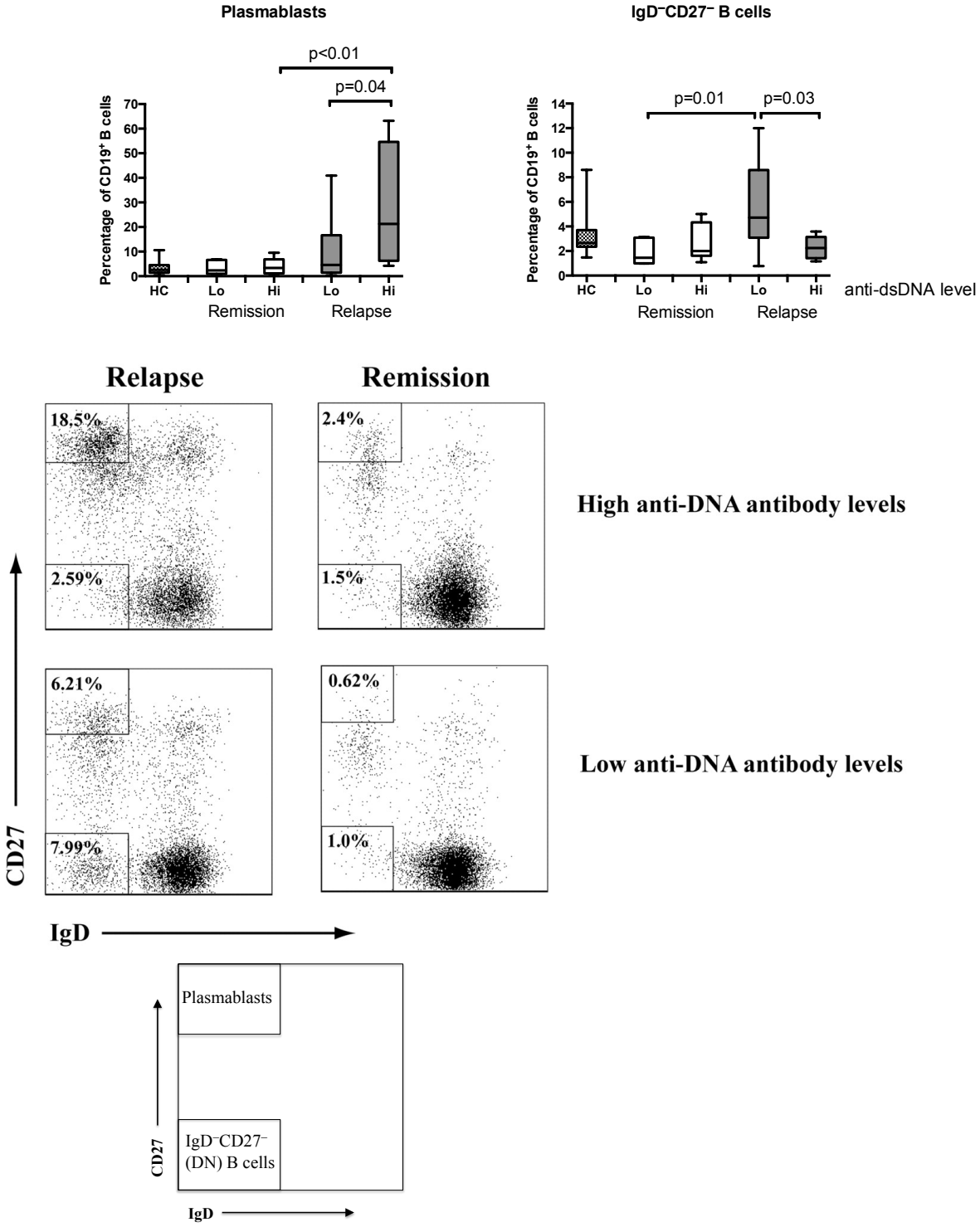
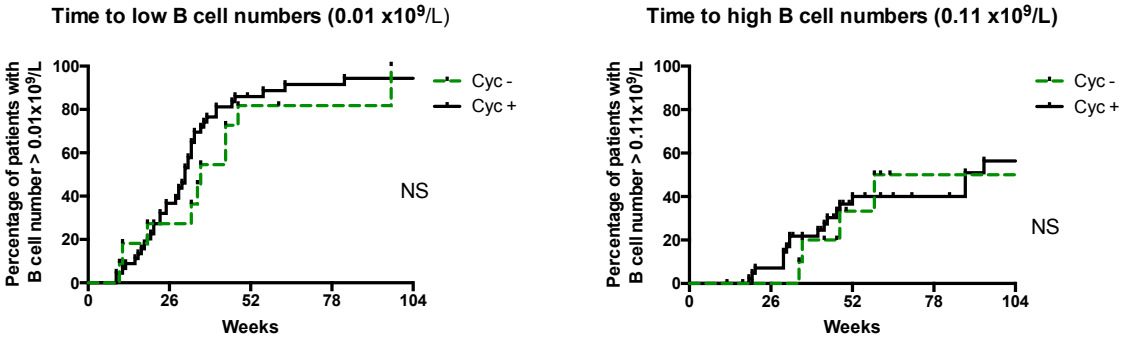


Figure 5.1.4 – B-cell subsets at clinical relapse in patients divided according to anti-dsDNA antibody levels

Peripheral blood B cells (gated on CD19) were analysed by flow cytometry for the expression of IgD and CD27. Representative and cumulative data are shown. Percentages of plasmablasts (IgD^-CD27^{hi}) or double-negative memory B cells (IgD^-CD27^-) in age- and sex-matched healthy controls [HC; n = 19] and in patients who are in remission or relapse with anti-dsDNA antibody levels that are low [lo (<100 IU/l); n = 7 (remission), n = 10 (relapse)] or high [hi (>100 IU/l); n = 9 (remission), n = 6 (relapse)] are indicated. Data are shown as box plots. Lines inside boxes indicate the median; outer borders of boxes indicate the 25th and 75th percentiles; bars extending from boxes indicate the range. Diffences between groups were analysed by Mann-Whitney U test.

A



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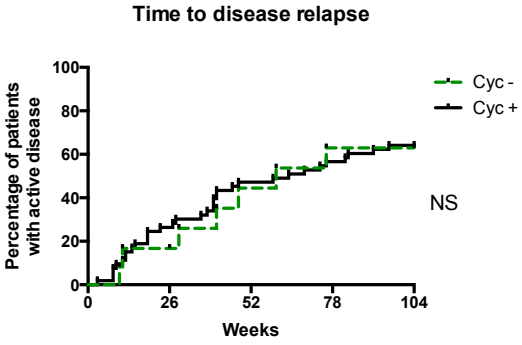


Figure 5.1.5 – Time to B cell repopulation and disease relapse following BCDT in patients given cyclophosphamide with rituximab compared to patients not given cyclophosphamide with rituximab

(**A**) Kaplan–Meier curves comparing time to B-cell repopulation $>0.01 \times 10^9$ B cells/l and $>0.11 \times 10^9$ B cells/l in patients given cyclophosphamide with rituximab [Cyc+; n = 45] and patients not given cyclophosphamide with rituximab [Cyc–; n = 11]. (**B**) Kaplan–Meier curve comparing time to disease relapse in patients given cyclophosphamide with rituximab [Cyc+; n = 53] and patients not given cyclophosphamide with rituximab [Cyc–; n = 12]. Comparisons of the curves were carried out using the log-rank test. NS = $p > 0.05$.

5.1.3 Discussion

Relapse following BCDT is associated with a combination of four key factors; differences in B cell repopulation rates, B cell numbers, two different B cell subsets and changes in anti-dsDNA antibody levels. How the B cell repopulation rate and B cell numbers affect relapse appears to depend on the last two factors. For example, relapse in patients with low anti-dsDNA antibody levels at the time of relapse appears to be partially dependent on the rate of repopulation of B cells to baseline levels and the main B cell is the $IgD^{-}CD27^{-}$ B cell subset. The rate of relapse in patients with high anti-dsDNA antibody levels is partially determined by how quickly B cells repopulate to low numbers ($>0.01 \times 10^{9}$ B cells/l) and the B cell associated with disease is the plasmablast. However, an additional variable needs to be considered in this last group, which is that their anti-dsDNA antibody levels might fall and this is also associated with a lower likelihood of relapsing, as has been observed in a previous study (435).

The data therefore identify three different groups;

1. Patients with low anti-dsDNA antibody levels that relapse with $IgD^{-}CD27^{-}$ B cells (or its precursor cell). The role of this B cell and its precursor cell are not known (Figure 5.1.6).

2. Patients with high anti-dsDNA antibody levels, whose response is associated with a fall in the anti-dsDNA antibody levels. It is likely that relapse is associated with the repopulation of the anti-dsDNA antibody producing plasma cells. The mechanisms that mediate the re-emergence of these B cell clones and the associated antibody producing plasma cells are not known, but will be examined later in this thesis.

3. Patients with high anti-dsDNA antibody levels that do not fall and in whom relapse is associated with low B cell numbers and plasmablasts. It has been suggested that anti-ENA antibodies arise from long-lived plasma cells (LLPC) and anti-dsDNA

antibodies arise from short lived plasma cells (SLPC) (286), but these data show that in a subset of patients there are also LLPCs that produce anti-dsDNA antibodies.

It is not known why some patients have SLPCs whilst others have LLPCs. Various mechanisms have been proposed for the differences in the lifespan of plasma cells, such as differences in where B and T cells interact, cytokine stimulation of B cells and where plasma cells find a survival niche, but most studies are from animal models and these mechanisms have not been proven in humans.

The next two chapters will examine factors that influence B cell repopulation and differentiation towards either plasmablasts or $IgD^{-}CD27^{-}$ B cells. Chapter 5.4 will then compare differences in histology between patients with low and high anti-dsDNA antibody levels. Chapter 5.5 will examine the mechanism of disease in patients with low anti-dsDNA antibody levels and the potential role of the $IgD^{-}CD27^{-}$ B cell in the disease. Chapters 5.6 and 5.7 will compare immunological and clinical factors between the three different patient groups separated according to their anti-dsDNA antibody levels as outlined above.

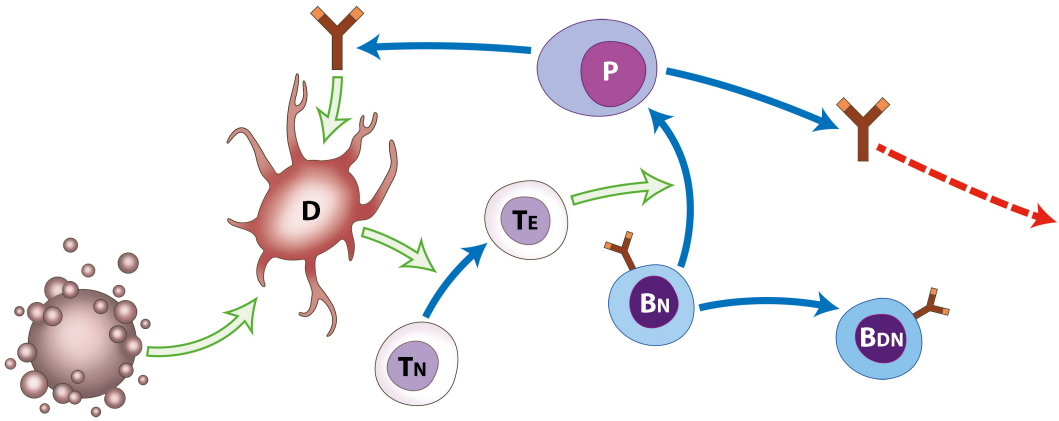


Figure 5.1.6 – Active SLE is associated with two B cell subsets
Naïve B cells (B_N) can differentiate into either plasmablasts or IgD^-CD27^- B cells (B_{DN}). Plasmablasts, which differentiate into plasma cells (P), are associated with active disease after BCDT in patients with high anti-dsDNA antibody levels, but IgD^-CD27^- B cells are associated with active disease after BCDT in patients with low anti-dsDNA antibody levels. The role of IgD^-CD27^- B cells in SLE and the mechanisms that induce their differentiation have not been established.

5.2 Memory T cell subsets predict the rate of B cell repopulation following BCDT in SLE

5.2.1 Introduction

$CD4^+$ T helper cells have been shown to have an important role in controlling the humoral immune response. However, they have also been shown to be important in the immune dyregulation that is seen in autoimmune diseases, which are often characterized by the presence of circulating autoantibodies.

T cells go through progressive stages of differentiation, which can be identified by the expression of the surface receptors CD45RA, CD28, CD27 and CCR7 (59). $CD4^+$ T cells that are $CD28^-CD27^-CCR7^-$ are considered to be the most terminally differentiated. CD45RA expression is mostly seen on naïve $CD4^+$ T cells but can also be seen on a small population of terminally differentiated T cells. There is evidence of increased terminal differentiation of $CD4^+$ T cells in several autoimmune diseases, including SLE (247, 248, 458), but the precise role of these memory T cells subsets in autoimmunity has not been fully elucidated.

In the previous chapter it was shown that the rates of B cell repopulation following BCDT in SLE vary between patients. In addition, there are two different B cell phenotypes that are associated with clinical relapse; IgD^-CD27^{hi} plasmablasts, in patients that relapse with high anti-dsDNA antibody levels, and IgD^-CD27^- memory B cells, in patients that relapse with low anti-dsDNA antibody levels. Whilst IgD^-CD27^- B cells have been shown to be atypical memory B cells (238), it is not known whether they share the same B cell precursor and originate from the same differentiation pathway as IgD^-CD27^+ memory B cells and plasmablasts, which are associated with antibody production.

$CD4^+$ memory T cell subsets have been shown to have differing effects on antibody synthesis by healthy B cells (459). We hypothesized that different memory T cell subsets might be associated with differences in B cell expansion and differentiation and account for some of

the differences in clinical response that have been observed following BCDT in patients with SLE.

5.2.2 Results

Memory T cell phenotypes do not change after BCDT

In chapter 5.2.1 it was observed that patients with low anti-dsDNA antibody levels experienced clinical relapse after BCDT with higher B cell numbers and had a high percentage of $IgD^{-}CD27^{-}$ memory B cells whilst patients with high anti-dsDNA antibody levels relapsed with lower B cell numbers and had a high percentage of $IgD^{-}CD27^{hi}$ plasmablasts. I therefore examined whether there were differences in T cell memory phenotypes in patients with either high or low anti-dsDNA antibody levels and assessed whether they are affected by the presence of B cells by measuring changes after BCDT.

Patients with active disease with low anti-dsDNA antibody levels (n = 16) had significantly lower percentages of naïve $CD4^{+}$ T cells in the $CD4^{+}$ T cell population than healthy controls (n = 19) ($p < 0.01$). There were no significant differences in the percentages of $CD45RA^{-}CD27^{+}$ central memory (Tcm), $CD45RA^{-}CD27^{-}$ effector memory (Tem) and $CD45RA^{+}CD27^{-}$ revertant memory (Trm) $CD4^{+}$ T cells between these two groups of patients, although there were three patients with low anti-dsDNA antibody levels with consistently high percentages of Tem and Trm cells (Figure 5.2.1). There were no statistically significant differences in the T cell memory phenotypes between patients with active disease and high anti-dsDNA antibody levels (n = 12) and healthy controls and between patients with active disease, with either low or high anti-dsDNA antibody levels, and patients with inactive disease (n = 26).

After BCDT the percentage of T cell memory phenotypes did not change at either week 6 or week 12 in the patients with low or high anti-dsDNA antibody levels, suggesting that B cells do not influence T cell differentiation.

Terminally differentiated $CD4^+CD27^-$ T cells are positively associated with IgD^-CD27^- (Double Negative) B cells and negatively associated with IgD^+CD27^+ non-switched memory B cells

$CD4^+$ T helper cells can influence B cell differentiation and antibody synthesis (460). I therefore hypothesized that terminally differentiated memory T cells would positively correlate with memory B cells and/or plasmablasts. However, the low percentage of naïve T cells in patients with low levels of anti-dsDNA antibodies suggests that terminally differentiated memory T cells might correlate with non-antibody producing B cells instead. To examine if terminally differentiated memory T cells correlate with one or more of the memory B cell subsets I compared the percentage of different B cell memory phenotypes from patients with SLE who had not previously been treated with rituximab (n = 28) and healthy controls (n = 12) with the percentage of terminally differentiated $CD4^+CD27^-$ T cells from the same time point.

The data show that there was a moderate positive correlation between the percentage of terminally differentiated $CD4^+CD27^-$ T cells and IgD^-CD27^- $CD19^+$ B cells ($r_p = 0.47$, $p = 0.002$) and a weak negative correlation between $CD4^+$ T cells and IgD^+CD27^+ $CD19^+$ B cells ($r_p = -0.38$, $p = 0.02$) that are non-switched memory B cells (Figure 5.2.2). There were no statistically significant correlations between $CD4^+CD27^-$ T cells and IgD^-CD27^{int} $CD19^+$ B cells (switched memory B cells) or IgD^-CD27^{hi} $CD19^+$ B cells (plasmablasts).

The relationship between Tem ($CD4^+CD45RA^-CD27^-$) and Trm ($CD4^+CD45RA^-CD27^-$) cell subsets and B cell subsets vary depending on the Trm:Tem ratio

Memory T cell subsets have different effects on B cell differentiation *in vitro* (459). Although both Tem and Trm cells are terminally differentiated they might have different functions (59). I therefore compared the relationship between the percentage of the different B cell subsets with the Trm:Tem ratio of patients with SLE who had not previously been treated with rituximab (n = 28) and healthy controls (n = 12). Figure 5.2.3 shows that there was a moderate positive correlation between the Trm:Tem ratio and IgD^-CD27^- $CD19^+$ B

cells (r_p = 0.47, p = 0.002) but there were no significant correlations between the Trm:Tem ratio and IgD^+CD27^+ B cells, IgD^-CD27^{int} $CD19^+$ B cells or IgD^-CD27^{hi} $CD19^+$ B cells. There appeared to be two distinct group of patients according to the Trm:Tem ratio. The relationship between the memory B cell subsets and Trm:Tem ratio appeared to differ in patients with a Trm:Tem ratio >0.15 compared to patients with a Trm:Tem ratio <0.15.

To investigate in more detail the relationship between the memory B cell subsets and the memory T cell subsets, Tem and Trm, the patients and healthy controls were divided into two groups according to whether they had a Trm:Tem ratio <0.15 (n = 33) or >0.15 (n = 7). There was a moderate positive correlation between Tem cells and IgD^-CD27^{hi} plasmablasts (r_p = 0.40, p = 0.02), a weak positive correlation between Tem cells and IgD^-CD27^- B cells (r_p = 0.39, p = 0.03) and a weak positive correlation between Trm cells and IgD^-CD27^{hi} plasmablasts (r_p = 0.36, p = 0.04) in the group with a low Trm:Tem ratio (<0.15) but not in the group with a high Trm:Tem ratio (Figure 5.2.4). However, there was a very strong positive correlation seen between Trm cells and IgD^-CD27^- B cells (r_p = 0.92, p = 0.004), a strong negative correlation between Trm cells and IgD^+CD27^+ non-switched memory B cells (r_s = −0.89, p = 0.01) and a strong negative correlation between Tem cells and IgD^+CD27^+ non-switched memory B cells (r_p = −0.78, p = 0.02) in the group with a high Trm:Tem ratio but not in the group with a low Trm:Tem ratio.

Terminally differentiated $CD4^+CD27^-$ T cells positively correlate with non-switched memory B cells and IgD^-CD27^- B cells after BCDT.

$CD4^+CD27^-$ T cells were correlated with B cell memory phenotypes from patients who had had BCDT (n = 39) in order to determine whether a similar relationship between B and T cell memory subsets occurred after B cells repopulate. A moderate positive correlation was seen again between $CD4^+CD27^-$ T cells and IgD^-CD27^- B cells (r_p = 0.35, p = 0.03), but there were no significant correlations seen between $CD4^+CD27^-$ T cells and the other memory B cell subsets (Figure 5.2.5).

I again carried out a subanalysis to examine the relationship between the memory B cell subsets and the memory T cell subsets, Tem and Trm, of the patients treated with BCDT who were divided into two groups according to whether they had a Trm:Tem ratio <0.15 (n = 34) or >0.15 (n = 5). There was a very strong positive correlation between Trm cells and $IgD^{-}CD27^{-}$ B cells ($r_p = 0.88$, $p < 0.05$) in patients with a high Trm:Tem ratio, but there were no other significant associations seen between the B cell subsets and the Tem or Trm subsets in patients with high or low Trm:Tem ratios (Figure 5.2.6).

The rate of B cell repopulation is higher in patients with high percentages of Trm cells.

If $CD4^{+}$ T cell memory subsets do not change after BCDT then the positive associations that are seen between the Tem with $IgD^{-}CD27^{hi}$ plasmablasts and $IgD^{-}CD27^{-}$ B cells and Trm cells with $IgD^{-}CD27^{-}$ B cells indicate that T cells are inducing B cell differentiation. $CD4^{+}$ T cells might therefore influence B cell expansion after BCDT and the differences in the percentage of $CD4^{+}$ T cell memory subsets might then be associated with different rates of B cell repopulation. In order to investigate possible differences in B cell repopulation patients were divided into a group that had high percentages of Trm cells (>0.8%), a group that had high percentages of Tem cells (>3%) but low/normal percentages of Trm cells and a group with low percentages of Tem cells.

B cells repopulate significantly faster in patients with high percentages of Trm cells (n = 10), followed by patients with high percentages of Tem cells but low percentages of Trm cells (n = 31) and the slowest in patients with low percentages of Tem cells (n = 13) ($p < 0.0001$) (Figure 5.2.7B). A difference in B cell numbers between patients with low percentages of Tem cells and patients with high percentages of Tem cells +/− high percentages of Trm cells became noticeable after approximately 20 weeks, whilst a difference in B cell numbers between patients with high percentages of Tem cells but low percentages of Trm cells and those patients with high percentages of Trm cells became noticeable after approximately 40 weeks.

Patients with high fractions of Trm cells relapse earlier

It has previously been shown that early B cell repopulation is associated with early relapse. It was therefore predicted that the time to clinical relapse would be reduced in patients with a high percentage of Trm cells, whose B cells repopulate at a faster rate, followed by patients with a high percentage of Tem cells but a low/normal percentage of Trm cells.

In parallel with the rates of B cell repopulation that were seen between the 3 groups, patients with high percentages of Trm cells (n = 11) relapsed the earliest, followed by patients with a high percentage of Tem cells but a low/normal percentage of Trm cells (n = 35) and then patients with low percentages of Tem cells (n = 13) ($P < 0.05$) (Figure 5.2.7C). The median time to relapse was 41 weeks for patients with a high percentage of Trm cells, 56 weeks for patients with a high percentage of Tem cells but a low percentage of Tem cells and over 104 weeks for patients with a low percentage of Tem cells. Similar to the differences in the rates of B cell repopulation the difference in the rates of relapse between patients with a low percentage of Tem cells and patients with a high percentage of Tem cells became noticeable at 29 weeks and the difference in the rates of relapse between patients with a high percentage of Tem cells but a low/normal percentage of Trm cells and patients with a high percentage of Trm cells became noticeable at 41 weeks. It is also notable that all but one of the 11 patients with a high percentage of Trm cells relapsed by 70 weeks.

In chapter 5.2.1 it was shown that patients with high levels of anti-dsDNA antibody levels relapse with lower numbers of B cells. I therefore hypothesized that patients with higher percentages of terminally differentiated T cells and high anti-dsDNA antibody levels would relapse earlier than patients with low anti-dsDNA antibody levels because the time to the B cell numbers required to relapse would be lower in the patients with high anti-dsDNA antibody levels. The results show that amongst patients with high percentages of Trm cells the patients with high anti-dsDNA antibody levels (n = 3) relapsed earlier than patients with low anti-dsDNA antibody levels (n = 8) ($p = 0.003$). There were no significant differences in the rates of relapse between patients with a high percentage of Tem cells but a low/normal

percentage of Trm cells with low anti-dsDNA antibody levels (n = 13) and patients with high anti-dsDNA antibody levels (n = 22) and between patients with a low percentage of Tem cells with low anti-dsDNA antibody levels (n = 7) and patients with high anti-dsDNA antibody levels (n = 6) (Figure 5.2.8).

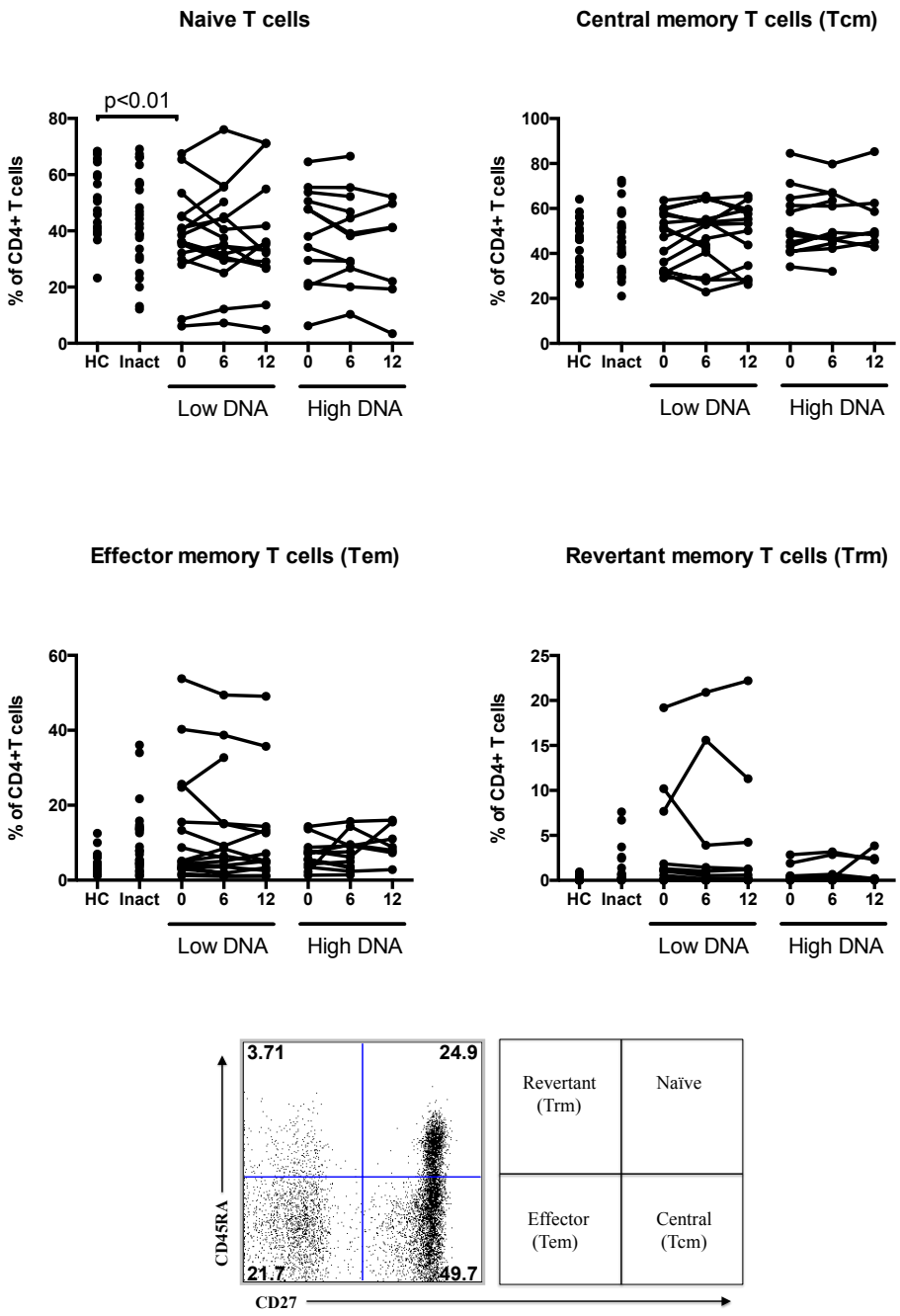


Figure 5.2.1 – Changes in CD4$^+$ T cell memory subsets after BCDT

Comparison of the percentage of CD4$^+$ T cell memory subsets in the peripheral CD4$^+$ T cell pool between healthy controls [HC; n = 19], patients with inactive SLE [Inact; n = 26] and active SLE with either low [Low DNA (<100 IU/L); n = 16] or high anti-dsDNA antibody levels [High DNA (>100 IU/L); n = 12] at baseline and following BCDT at weeks 6 and 12. Results are shown for CD4$^+$CD45RA$^+$CD27$^+$ naïve T cells, CD4$^+$CD45RA$^-$CD27$^+$ central memory T cells (Tcm), CD4$^+$CD45RA$^-$CD27$^-$ effector memory T cells (Tem) and CD4$^+$CD45RA$^+$CD27$^-$ revertant memory T cells (Trm). Representative and cumulative data are shown. Differences between groups were analysed by the Mann-Whitney U test for unpaired data or the Wilcoxon signed rank test for paired data.

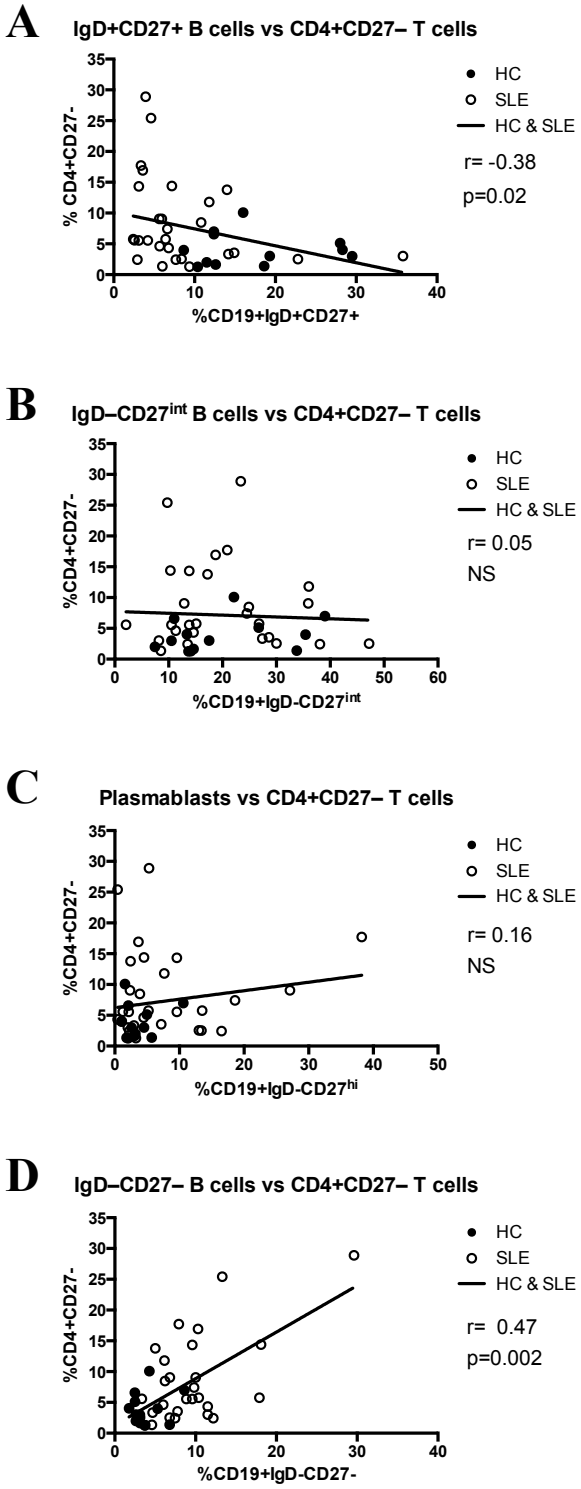


Figure 5.2.2 – Relationship between the percentage of $CD4^+CD27^-$ memory T cells in the circulating $CD4^+$ T cell pool and the percentages of $CD19^+$ B cell memory subsets before BCDT

$CD19^+$ B cell memory subsets were identified using the surface markers IgD and CD27; non-switched memory, IgD^+CD27^+ (**A**), switched-memory, IgD^-CD27^{int} (**B**), plasmablasts, IgD^-CD27^{hi} (**C**) and double-negative memory, IgD^-CD27^- (**D**). The data from healthy controls [n = 12] and patients with SLE who had not been treated with rituximab [n = 28] are represented as scatter plots and lines of best fit (for healthy controls and SLE patients combined). The Pearson correlation coefficient (r_p) is provided for each association. NS = $p > 0.05$.

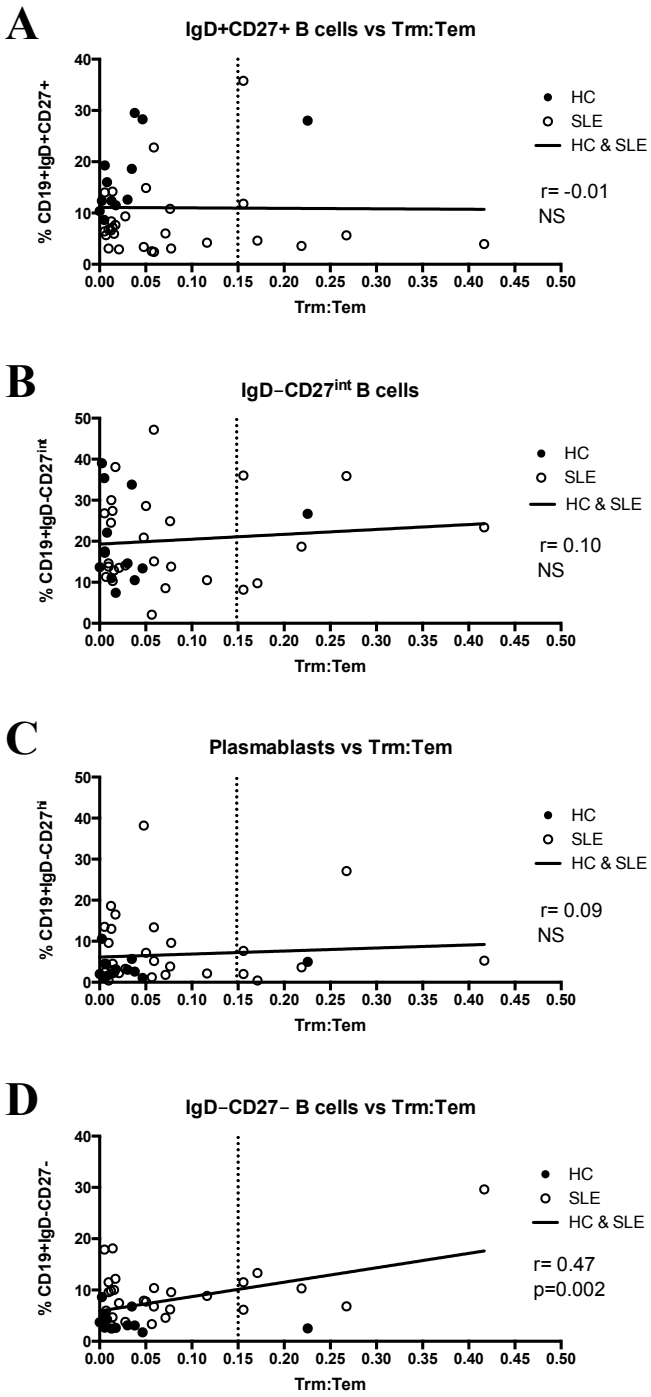


Figure 5.2.3 – Relationship between the ratio of $CD4^+CD45RA^+CD27^-$ (Trm) and $CD4^+$-$CD45RA^-CD27^-$ (Tem) cells in the circulating $CD4^+$ T cell pool and the percentages of $CD19^+$ B cell memory subsets before BCDT

$CD19^+$ B cell memory subsets were identified using the surface markers IgD and CD27; non-switched memory, IgD^+CD27^+ (**A**), switched-memory, IgD^-CD27^{int} (**B**), plasmablasts, IgD^-CD27^{hi} (**C**) and double-negative memory, IgD^-CD27^- (**D**). The data from healthy controls [n = 12] and patients with SLE who had not been treated with rituximab [n = 28] are represented as scatter plots and lines of best fit (for healthy controls and SLE patients combined). The vertical dotted line shows the separation of patients with a low Trm:Tem ratio (<0.15) from patients with a high Trm:Tem ratio (>0.15). The Pearson correlation coefficient (r_p) is provided for each association. NS = $p > 0.05$.

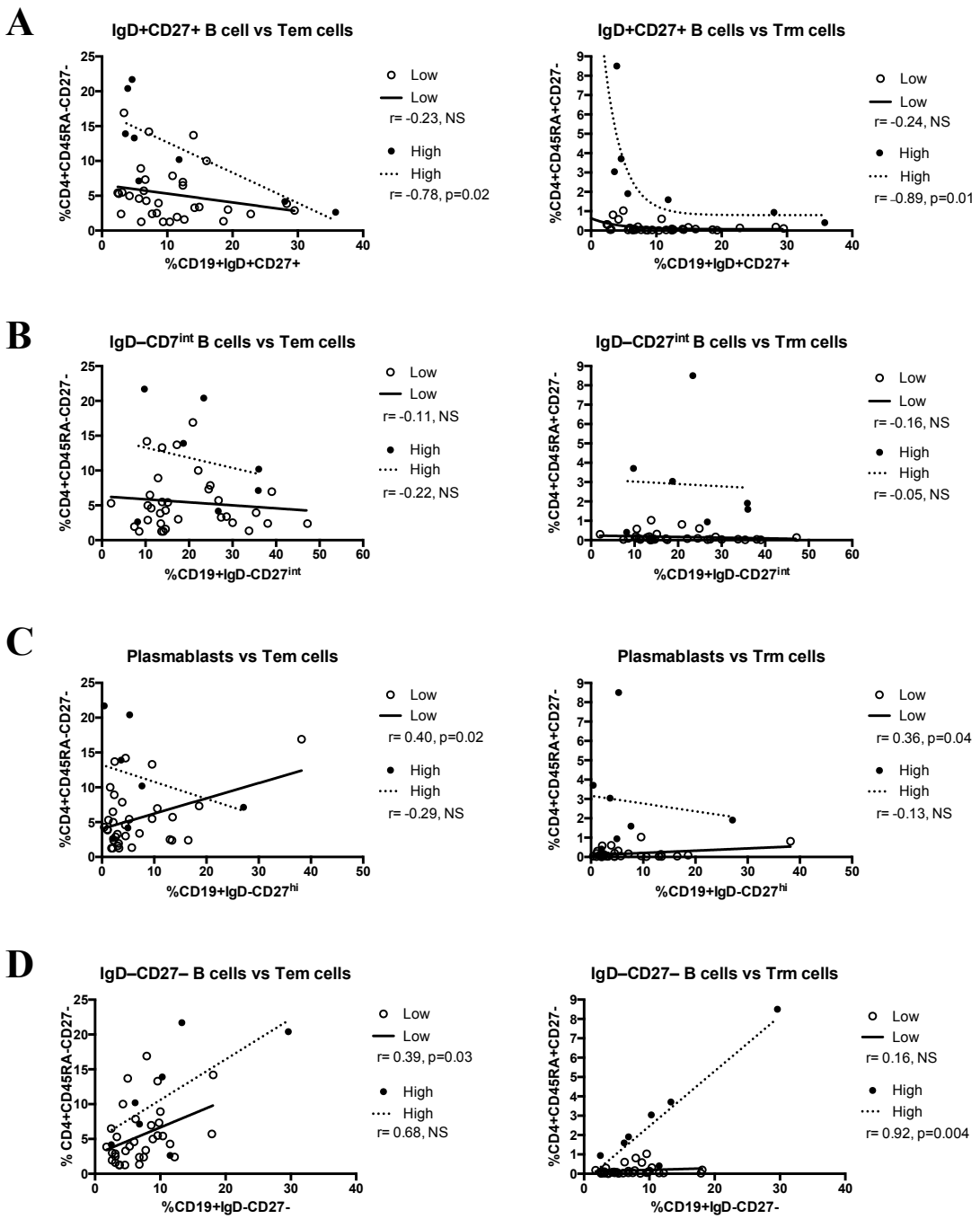


Figure 5.2.4 – Relationship between the percentages of $CD4^+CD45RA^-CD27^-$ effector memory T cells (Tem) and $CD4^+CD45RA^+CD27^-$ revertant memory T cells (Trm) in the circulating $CD4^+$ T cell pool and the percentages of $CD19^+$ B cell memory subsets before BCDT

$CD19^+$ B cell memory subsets were identified using the surface markers IgD and CD27; non-switched memory, IgD^+CD27^+ (**A**), switched-memory, IgD^-CD27^{int} (**B**), plasmablasts, IgD^-CD27^{hi} (**C**) and double-negative memory, IgD^-CD27^- (**D**). The data from individuals (healthy individuals and patients with SLE who had not been treated with BCDT) with high Trm:Tem ratios [High (>0.15); n = 7] and with low Trm:Tem ratios [Low (<0.15); n = 33] are represented as scatter plots and lines of best fit. The Pearson correlation coefficient (r_p) is provided for linear relationships and Spearman Rank correlation coefficient (r_s) is provided for curvilinear relationships. NS = $p > 0.05$.

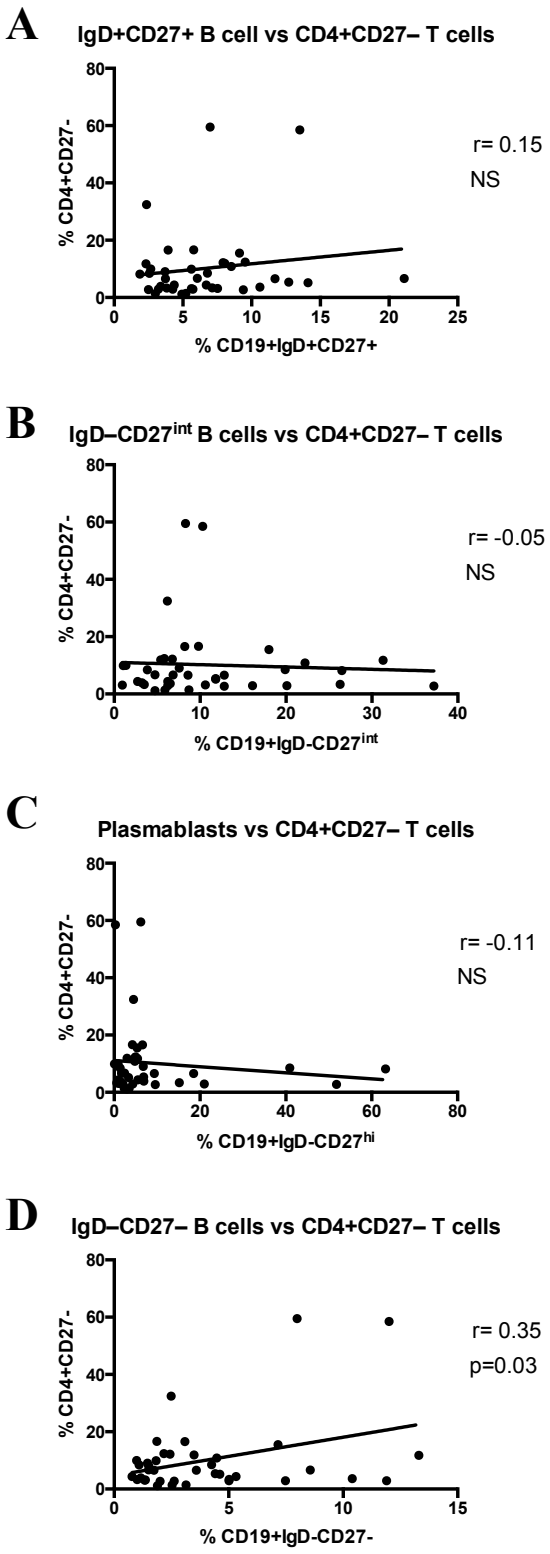


Figure 5.2.5 – Relationship between the percentage of $CD4^+CD27^-$ memory T cells in the peripheral $CD4^+$ T cell pool and the percentages of $CD19^+$ B cell memory subsets after BCDT

$CD19^+$ B cell memory subsets were identified using the surface markers IgD and CD27; non-switched memory, IgD^+CD27^+ (**A**), switched-memory, IgD^-CD27^{int} (**B**), plasmablasts, IgD^-CD27^{hi} (**C**) and double-negative memory, IgD^-CD27^- (**D**). The data from patients with SLE who have been treated with BCDT [n = 39] are represented as scatter plots and lines of best fit. The Pearson correlation coefficient (r_p) is provided for each association. NS = $p > 0.05$.

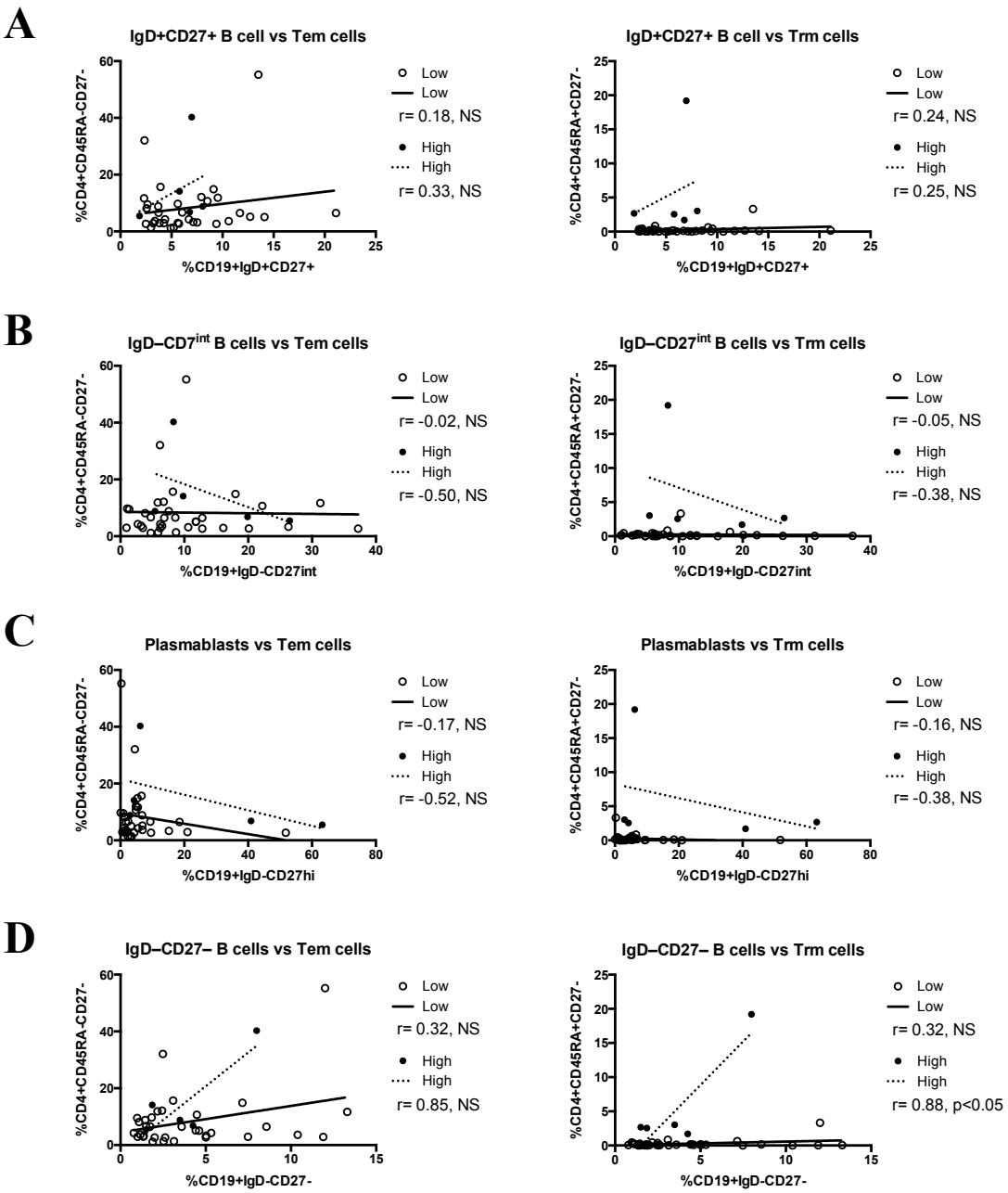


Figure 5.2.6 – Relationship between the percentages of $CD4^+CD45RA^-CD27^-$ effector memory T cells (Tem) and $CD4^+CD45RA^+CD27^-$ revertant memory T cells (Trm) in the circulating $CD4^+$ T cell pool and the percentages of $CD19^+$ B cell memory subsets after BCDT

$CD19^+$ B cell memory subsets were identified using the surface markers IgD and CD27; non-switched memory, IgD^+CD27^+ (**A**), switched-memory, IgD^-CD27^{int} (**B**), plasmablasts, IgD^-CD27^{hi} (**C**) and double-negative memory, IgD^-CD27^- (**D**). The data from individuals (healthy individuals and patients with SLE who had not been treated with BCDT) with high Trm:Tem ratios [High (>0.15); n = 5] and with low Trm:Tem ratios [Low (<0.15); n = 34] are represented as scatter plots and lines of best fit. The Pearson correlation coefficient (r_p) is provided for linear relationships and Spearman Rank correlation coefficient (r_s) is provided for curvilinear relationships. NS = $p > 0.05$.

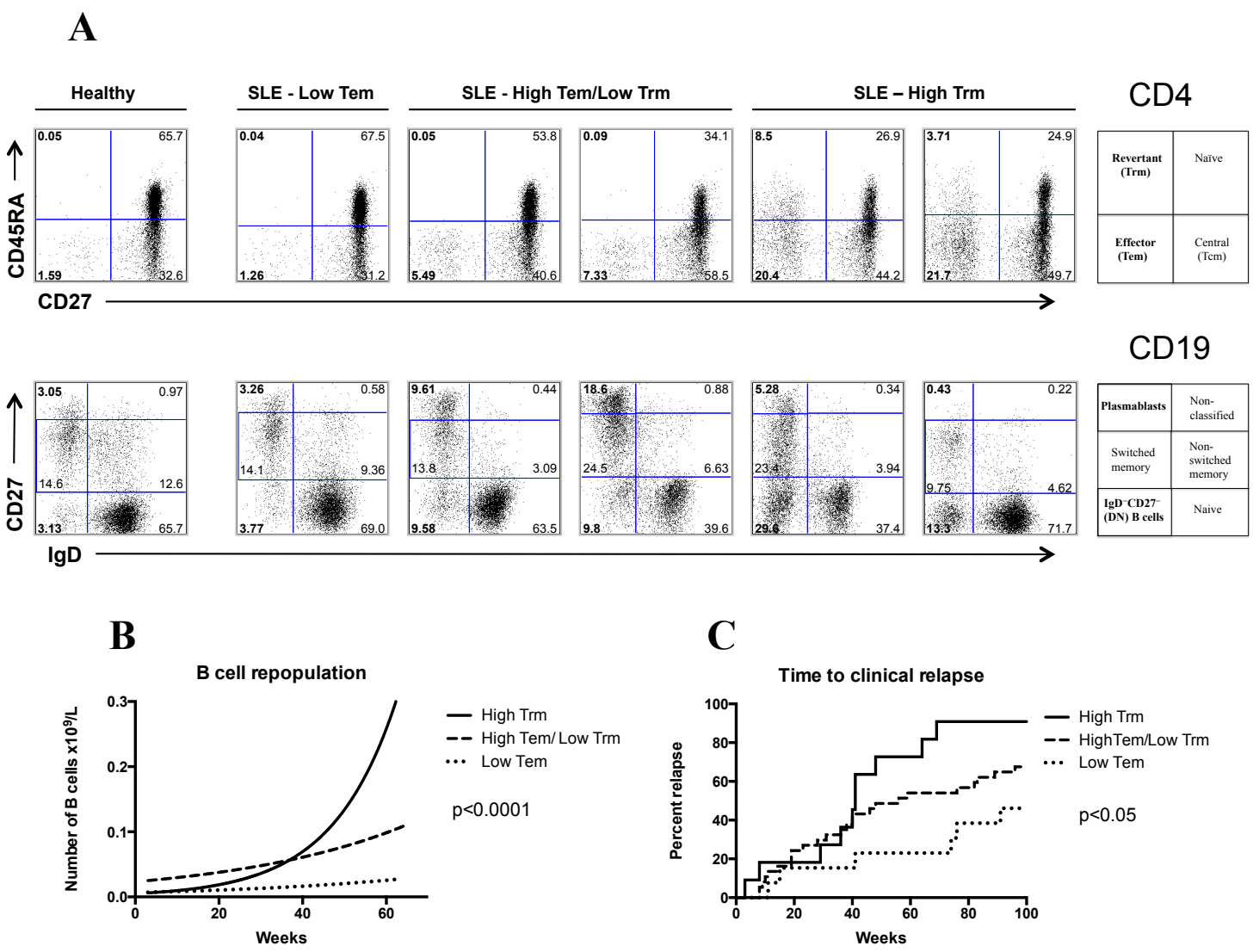


Figure 5.2.7 – Comparison of the rates of B cell repopulation and clinical relapse according to memory T cell phenotypes

(**A**) Examples of two-parameter dot plots obtained by flow cytometry from one healthy individual and five rituximab-naive SLE patients. The dot plots illustrate the relationship between $CD4^+$ memory T cells, $CD4^+CD45RA^-CD27^-$ (Tem) and $CD4^+CD45RA^+CD27^-$ (Trm), and the B cell subsets, $CD19^+IgD^-CD27^{hi}$ plasmablasts and $CD19^+IgD^-CD27^-$ double negative (DN) memory B cells (gate percentages are shown in bold). Three SLE groups are shown; 1. Tem cells <3% (Low Tem) associated with low percentages of plasmablasts and IgD^-CD27^- B cells, 2. Tem cells >3% but Trm cells <0.8% (High Tem/ Low Trm) associated with high percentages of plasmablasts and IgD^-CD27^- B cells, 3. Tem cells >3% and Trm cells >0.8% (High Trm) associated with low percentages of plasmablasts but high percentages of IgD^-CD27^- B cells. (**B**) Comparison of the changes in B cell numbers after BCDT using repeated measures ANOVA. Curves show the circulating B cell numbers over time of SLE patients who had been treated with BCDT, divided into 3 groups; Low Tem [n = 13], High Tem/ Low Trm [n = 31] and High Trm [n = 10]. The lines were fitted using non-linear regression and compared using the extra sum-of-squares F test. (**C**) Kaplan-Meier estimates of the time to clinical relapse following BCDT according to the 3 groups; Low Tem [n = 13], High Tem/ Low Trm [n = 35], High Trm [n = 11]. Differences were tested using the log-rank test for trend.

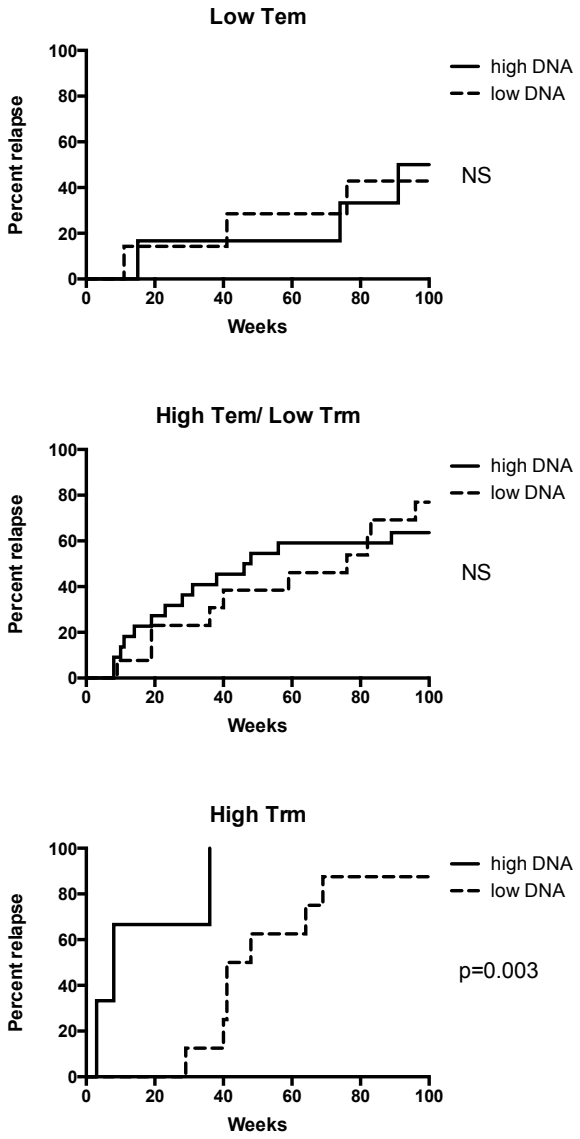


Figure 5.2.8 – Comparison of the rates of clinical relapse according to memory T cell phenotypes and anti-dsDNA antibody levels

Kaplan-Meier estimates of the time to clinical relapse following BCDT in patients with Tem cells <3% (Low Tem) with anti-dsDNA antibody levels >100 IU/L [high DNA; n = 6] or <100 IU/L [low DNA; n = 7], Tem cells >3% but Trm cells <0.8% (High Tem/ Low Trm) with anti-dsDNA antibody levels >100 IU/L [high DNA; n = 22] or <100 IU/L [low DNA; n = 13] and Tem cells >3% and Trm cells >0.8% (High Trm) with anti-dsDNA antibody levels >100 IU/L [high DNA; n = 3] or <100 IU/L [low DNA; n = 8]. Differences were tested using the log-rank test for trend. NS = $p > 0.05$.

5.2.3 Discussion

Rituximab depletes the blood and SLOs of $CD20^+$ mature B cells but not the B cell precursors, which do not express the surface marker CD20. The mature B cell compartment is repleted either by proliferation of B cells that have survived following treatment, possibly by being protected by the microenvironment or due to resistance of the B cell to rituximab induced cell death (461), or they emerge from the production of new immature B cells within the bone marrow (lymphopoiesis) followed by selection and maturation of the B cells in the SLOs (differentiation) and then activation of mature B cells.

$CD4^+$ memory T cells can be found in the bone marrow (462), but whilst they have been shown to influence B cell differentiation (459), they have not, to my knowledge, been shown to have a direct effect on B lymphopoiesis. It is, therefore, possible that the $CD4^+$ memory T cells that are present in the bone marrow prevent the deletion of naïve autoreactive B cells to allow them to migrate to SLOs, where they then induce B cell maturation and then activation and expansion of memory B cells from the naïve B cell pool. In lupus-prone mice $CD4^+$ T cells have been shown to play an important role in the clonal expansion of autoreactive B cells and autoantibody secretion (203, 463), probably as a result of IL-4, IL-10, IFN-γ and/or CD40 stimulation, depending on the mouse strain. Antigen-specific cognate interactions between B and $CD4^+$ T cells might not be necessary. In one strain of lupus-prone mice the interaction between $CD4^+$ T cells and autoreactive B cells was shown to occur in the follicles of the spleen (464).

Similar mechanisms of T-cell induced B cell activation have also been shown in human studies. For example, B cells from patients with SLE produce autoantibodies in response to CD40 stimulation by T cells (237). T cells from patients with SLE can also produce the B cell stimulating cytokine, BAFF, which is elevated in the serum of patients with SLE, both directly (267, 465) and indirectly by stimulating peripheral blood monocytes with IFN-γ (466). BAFF plays an important role in rescuing newly formed autoreactive B cells and allowing them to enter normally restricted niches in SLOs (467, 468). Inhibition of BAFF in

patients with SLE leads to a decrease in naïve and transitional B cells initially, then memory B cells and plasmablasts accompanied by a decline in serum IgM levels (469). T-cell derived IL-6 might also be important for polyclonal B cell activation and autoantibody production (470), supported by observations that IL-6 blockade leads to a reduction in $IgD^{-}CD27^{+}$ memory B cells and an increase in $IgD^{+}CD27^{-}$ antigen-inexperienced mature B cells (471). In contrast to BAFF blockade, IL-6 blockade leads to an increase in naïve B cells, possibly because IL-6 induces the differentiation of pre-activated B cells, i.e. a later stage of B cell development (472).

It would be useful to know if the high percentage of circulating Tem and Trm cells in patients with SLE correlate with higher levels of these T cells in the bone marrow, where they have been found to preferentially localize (462), as this might provide a mechanism for how immature naïve autoreactive B cells are rescued from deletion and a possible explanation for the higher rate of B cell expansion that is seen in patients with high percentages of Trm cells. However, IL-7, produced by stromal cells in the bone marrow, has been shown to induce both B lymphopoiesis and the differentiation of Tem cells to Trm cells (462, 473). It is therefore possible that the higher rate of B cell expansion that is seen in patients with high percentages of Trm cells is due to a shared microenvironment rather than due to direct interaction between B and T cells. However, as the percentage of $CD4^{+}$ memory T cell subsets does not change after BCDT and that a similar correlation between $CD4^{+}CD27^{-}$ T cells and $IgD^{-}CD27^{-}$ B cells is seen both before and after BCDT the most likely explanation is that these memory T cell subsets are influencing the differentiation of B cells.

My data suggest that Tem and Trm cells have different roles in the activation of mature B cells; Tem cells positively correlate with $IgD^{-}CD27^{-}$ B cells and $IgD^{-}CD27^{+}$ memory B cells and plasmablasts, although only when there is a low Trm:Tem ratio, and Trm cells positively correlate with $IgD^{-}CD27^{-}$ memory B cells and are negatively associated with $IgD^{+}CD27^{+}$ memory B cells. These two memory T cell subsets have been shown to have slightly different

functions (462). Tem cells are more likely to be activated and appear to express higher levels of CD40 ligand, which might explain their positive association with plasmablasts.

The role and function of $IgD^{-}CD27^{-}$ B cells in the disease is unknown but this study shows that they might arise from a different differentiation pathway to $IgD^{-}CD27^{int}$ memory B cells, via stimuli from different $CD4^{+}$ memory T cell subsets, and it would therefore support the hypothesis that they have different roles in the disease. They are increased in healthy elderly individuals (474) and healthy individuals exposed to respiratory syncytial virus (277), which supports a relationship with Trm cells, as they are also increased in the elderly (475) and following persistent viral infection (462). Phenotypic studies of elderly individuals show that $IgD^{-}CD27^{-}$ B cells express IgG but express low levels of CD80 and HLA-DR suggesting that they do not act as APCs. They also do not express significant levels of CD40. In SLE, these cells have not been shown to have any specific function and it is unclear whether they have a role in active disease (238, 271).

Whilst studies have shown that $IgD^{-}CD27^{-}$ B cells are increased in SLE $IgD^{+}CD27^{+}$ B cells are reduced (277). This observation is supported by the finding that there is a significant negative association between $IgD^{-}CD27^{-}$ B cells and $IgD^{+}CD27^{+}$ B cells ($r_p = -0.31$, $p = 0.04$), whilst there is a positive association between $IgD^{-}CD27^{int}$ switched memory B cells and $IgD^{-}CD27^{hi}$ plasmablasts ($r_p = 0.46$, $p = 0.002$) (data not shown). There is no association between $IgD^{-}CD27^{int}$ switched memory B cells or $IgD-CD27^{hi}$ plasmablasts and $IgD^{-}CD27^{-}$ B cells. It is therefore possible that there are two distinct differentiation pathways, that are mediated by differences in the percentages of Tem and Trm cells.

Although Tem cells correlate with plasmablasts $CD4^{+}$ memory T cell subsets do not differ significantly in patients with high or low anti-dsDNA antibodies, suggesting that $CD4^{+}$ T cells do not necessarily influence anti-dsDNA antibody synthesis. However, the presence of anti-dsDNA antibodies is associated with a faster rate of relapse in patients with high percentages of Trm cells, suggesting that anti-dsDNA antibodies might have a role in

exacerbating the pathogenic function of $CD4^+$ T cells. As $CD4^+$ T cells do not express Fc receptors, this is likely to be an indirect role.

Despite the various studies that show how T cells can influence B cell function, I am unaware of any study in mice or humans, which show that $CD4^+$ effector and/or revertant memory T cell subsets can influence the differentiation of memory B cell subsets *in vivo*. One study has shown that Tem and Trm function differs *in vitro* (462), but there have been no *in vivo* functional studies, which is important because stimulating resting T cells *in vitro* might be different to antigen activated cells *in vivo*. Similarly, there have been no studies, which have looked at how T cell function differs when there are different Trm:Tem cell ratios.

The results show that there are three distinct patterns of B cell expansion following BCDT, which are associated with different percentages of circulating $CD4^+$ memory T cell subsets. Patients with high percentages of Tem cells but low/normal percentages of Trm cells have higher numbers of B cells as early as week 2 but they do not rise as rapidly as the B cell numbers of patients with high percentages of Trm cells after week 20, whilst patients with low percentages of Tem and Trm cells do not see a substantial rise in B cell numbers for at least one year. The higher B cell numbers at week 2 of patients with high percentages of Tem cells but low percentages of Trm cells might be due to inadequate depletion of B cells but I cannot provide an explanation for how this could be due to Tem cells. A more likely explanation is provided by the observation that Tem cells are associated with plasmablasts, suggesting that this early repopulation is due to the production of small numbers of plasmablasts, which has been seen in a group of patients with a higher risk of relapse within the first 18 months (429). The B cell numbers (measured using the CD19 marker) of the patients with high percentages of Tem cells but low/normal percentages of Trm cells might not appear to rise as rapidly as the group with high percentages of Trm cells after week 20 because the plasmablasts are differentiating into $CD19^-$ plasma cells, which are not included in the B cell count. Trm cells are associated with IgD^-CD27^- B cells rather than plasmablasts and IgD^-CD27^- B cells have not been shown to differentiate into antibody producing plasma

cells. It is therefore likely that this rapid rise in B cell numbers in the patients with high percentages of Trm cells after week 20 is partly due to a steady accumulation of $IgD^{-}CD27^{-}$ B cells. However, the B cell expansion that is seen in the patients with high percentages of Trm cells cannot only be attributed to a rise in $IgD^{-}CD27^{-}$ B cells because they only make up 4.1% (mean) of the B cell population, suggesting that other B cell subsets are also expanding.

My data indicate that long-term control of disease is more likely if T cells are less differentiated. The mechanisms that influence T cell differentiation might be useful therapeutic targets but they are currently unclear. Certain factors that have been shown to be important include TCR engagement with antigen and stimulation by pro-inflammatory cytokines such as IFN-α and IL-6, both of which are known to be high in SLE as a result of immune complex activation of PDCs. Interestingly, IL-6 blockade leads to an increase in $CD4^{+}CD45RA^{+}CCR7^{+}$ naïve T cells (471), suggesting that this might be a possible strategy for inducing long-term disease remission.

A model of disease is proposed where immune complexes bound to protein containing RNA induce PDCs to express cytokines, probably IFN-α and/or IL-6, which cause the differentiation of $CD4^{+}$ T cells into memory T cell subsets, which in turn influence B cells to differentiate into either plasmablasts or $IgD^{-}CD27^{-}$ B cells (Figure 5.2.9). The questions that result from this study are what factor leads to the preferential differentiation of T cells to Trm or Tem, which factors influence the differentiation of B cells towards either plasmablasts or $IgD^{-}CD27^{-}$ B cells and what is the role of $IgD^{-}CD27^{-}$ B cells in the disease.

Finally, this study shows that the rate of B cell repopulation matches the rate of clinical relapse supporting evidence that B cells have an important role in disease. The different rates of B cell repopulation might partially explain the heterogeneity of the disease, in particular the differences in clinical response to B cell targeted therapy. Results of future clinical trials might be improved by stratifying patients according to their circulating $CD4^{+}$ T cell memory subsets and anti-dsDNA antibody levels.

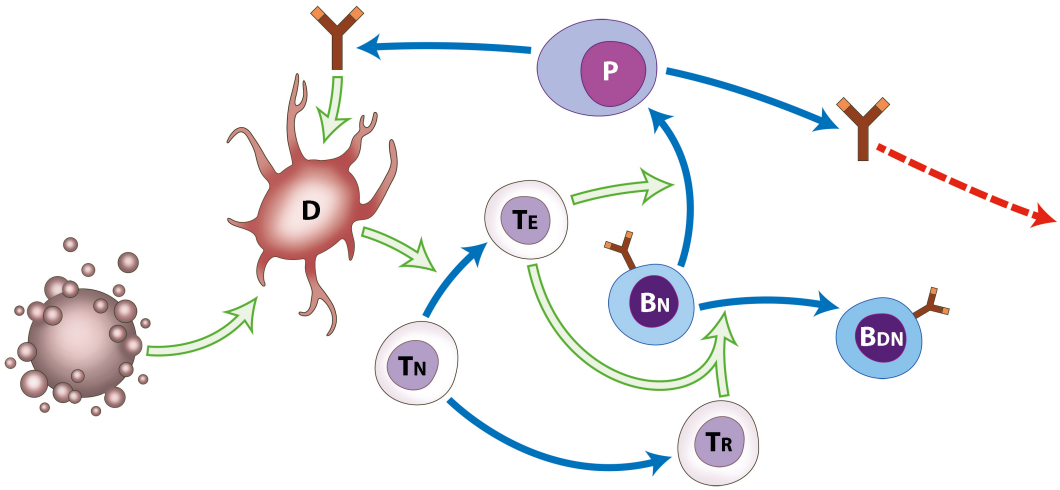


Figure 5.2.9 – Plasmablasts and IgD$^-$CD27$^-$ B cells correlate with different memory T cells

Plasmablasts (P) correlate with CD4$^+$CD45RA$^-$CD27$^-$ effector T cells (T_E) and IgD$^-$CD27$^-$ B cells (B_{DN}) correlate with CD4$^+$CD45RA$^-$CD27$^-$ effector T cells (T_E) and CD4$^+$CD45RA$^+$CD27$^-$ revertant T cells (T_R). Memory T cells do not change after BCDT, therefore suggesting that these T cells induce the differentiation of B cells.

5.3 Pregnancy might induce B cell repopulation after BCDT in SLE

5.3.1 Introduction

Chapter 5.1 showed an association between B cell repopulation and clinical relapse following BCDT in SLE. Chapter 5.2 showed that memory T cells might influence B cell differentiation and expansion in some patients, but other factors are also likely to influence B cells. The increased incidence of SLE in young women (118) and the possible increase in disease flares during pregnancy (119) suggest that female hormones influence lymphocyte numbers and/or function.

Circulating B cell numbers have been shown to either remain stable (476) or fall during healthy pregnancy (477, 478), but I am not aware of any study that has examined the effect of pregnancy on circulating B cell numbers in patients with SLE. The cases of four patients who became pregnant at least one year after BCDT are described. Three of these patients developed disease flares shortly after becoming pregnant. The changes in B cell numbers, anti-dsDNA antibody and C3 levels following BCDT, during pregnancy and flare are described and shown in Figure 5.3.1.

5.3.2 Case Histories

Patient 47 is a 28-year-old Indian woman who was treated with her first cycle of rituximab for arthritis and cutaneous disease. She was positive for Ro, Sm and RNP antibodies and negative for lupus anticoagulant and anticardiolipin antibodies. Her anti-dsDNA antibody level was 203 IU/Land C3 level was 0.69 g/L prior to therapy. After rituximab therapy her symptoms resolved even though her anti-dsDNA antibody levels transiently rose above the pre-treatment level on two separate occasions. Her C3 steadily increased to normal levels. She became pregnant 96 weeks after rituximab therapy. It was her third pregnancy. Her first child was born five years previously, with no significant foetal complications. She was induced and subsequently had an emergency Caesarean section for presumed foetal distress. However, she had a lupus flare in the post-partum period. Her second pregnancy was terminated two years previously because her SLE was active and shortly afterwards she was treated with rituximab. At the time of conception she was taking azathioprine 100 mg, hydroxychloroquine 400 mg and prednisolone 15 mg.

From B cell depletion to conception her B cell count was less than 0.005 x 10^9/L but was 0.01 x 10^9/L 13 weeks after the estimated time of conception. This small rise in her B cell count coincided with a disease flare with symptoms of chest and back pain, thought to be due to serositis, arthralgia and a malar rash (3 x BILAG B scores). During this flare her anti-dsDNA antibody level was 164 IU/L, which was elevated but lower than previous measurements. Her C3 level was 0.88 g/L and had not fallen significantly. Her symptoms settled with 30 mg of oral prednisolone. There were no further complications during her pregnancy and she delivered a healthy baby, weighing 2350 g, at 38+3 weeks. Three weeks after she gave birth her B cell count was 0.111 x 10^9/L and continued to rise to a peak level of 0.181 x 10^9/L. She had two further disease flares, one six months (fever, lymphadenopathy, rash; 2 x BILAG A scores) and the other 18 months after labour (fever, arthralgia; 2 x BILAG B scores). Both flares required admission to hospital, and both responded to intravenous methylprednisolone.

During each flare of lupus her anti-dsDNA level was elevated, although had not increased prior to or during the flare and below the pre-rituximab level. Her C3 levels remained normal.

Patient 10 is a 38-year-old Afro-Caribbean woman with a history of class IV glomerulonephritis, arthritis and cutaneous disease. She was positive for Sm and RNP antibodies and negative for lupus anticoagulant and anticardiolipin antibodies. She had had three previous pregnancies, with one live birth at 34 weeks by spontaneous vaginal delivery and two previous early miscarriages. Her previous successful pregnancy was complicated by possible pre-eclampsia or flare of lupus nephritis.

She was treated with her fourth cycle of rituximab. Her anti-dsDNA antibody level was 120 IU/L and C3 level was 0.83 g/L prior to therapy. After therapy her urinary protein to creatinine ratio (PCR) fell from 156 to 92 and her symptoms improved. Her anti-dsDNA antibody level fell to normal levels and the C3 level also increased. She became pregnant for the fourth time 78 weeks after her first rituximab infusion.

From B cell depletion to conception her B cell count was less than 0.01 x 10^9/L. Ten weeks after the estimated date of conception her B cell count was 0.012 x 10^9/L and continued to rise both during and after pregnancy to a peak level of 0.079 x 10^9/L at 62 weeks after conception and then fell to 0.029 x 10^9/L.

The second pregnancy was complicated by thyrotoxicosis, microcytic anaemia (Hb 8.8) and hypertension. Blood pressure (BP) at booking was 160/85, which was treated with nifedipine 10 mg once per day (od). Her SLE was controlled with prednisolone 5 mg od. She was commenced on aspirin and ferrous sulphate 10 weeks into her pregnancy. At 11 weeks, she was noted to have mild leg tremors, minimal exopthalmos and a small diffuse thyroid. She was therefore started on propylthiouracil (PTU) 100 mg twice per day (bd) and propranolol 40 mg three times per day (tds). The symptoms of thyrotoxicosis subsided, and six weeks later her PTU was reduced to 50 mg bd and her propranolol stopped. Her blood pressure rose to 142/92 at 19 weeks, attributed to stopping propranolol. However this remained high

(167/97) and the nifedipine dose was doubled to 20 mg bd. Review at 20 weeks noted her lupus to be quiescent. At 23 weeks she developed proteinuria 2+, which increased over the next three weeks to 4+. This was associated with mild bilateral leg swelling. Her blood pressure remained stable, and she had no other pre-eclampsia symptoms. At 30 weeks her serum creatinine had inceased to 134, and the PCR increased to 194. Her nifedipine was increased to 30mg bd. A plan was made after 32 weeks to consider early delivery if the serum creatinine rose above 170 to prevent irreparable damage to maternal renal function. Over the next 2 weeks her serum creatinine increased to 160, and PCR rose to 404. Thromboprophylaxis and labetolol 100 mg tds were introduced at 34 weeks, and her BP remained below 135/85. She continued to have heavy proteinuria but her blood test results and symptoms were not suggestive of pre-eclampsia. Labour was induced at 35+4 weeks because of the increasing serum creatinine. A live infant was delivered by ventouse, weighing 1899 g.

After the pregnancy she continued to require anti-hypertensive medication. Her PCR peaked at 665 at 27 weeks after the pregnancy and then fell to between 169 and 275. Her creatinine did not improve after the pregnancy and continued to rise steadily. Her C3 level fell 9 weeks after labour (44 weeks after conception) from 1.44 to 0.93 g/L. At 88 weeks her anti-dsDNA antibody level increased from 27 to 127 IU/L, which was associated with a rise in her creatinine from 187 to 306 (1 x BILAG A score). Her prednisolone dose was then increased to 20 mg and she was also commenced on Mycophenolate Mofetil, which resulted in her anti-dsDNA antibody levels returning to normal and a small improvement in her creatinine.

Patient 57 is a 34-year-old Afro-Caribbean woman with a history of arthralgia, photosensitivity, a malar rash, glomerulonephritis with minimal change on biopsy and neuropsychiatric lupus. She was positive for Sm and RNP antibodies and negative for lupus anticoagulant and anticardiolipin antibodies. She had had three previous pregnancies. Her first pregnancy was terminated when she was 15 years old, she then had a miscarriage at 24 years of age, followed by a live pregnancy at 33/40 when she was 30 years old. This

pregnancy followed a period of subfertility, due to being unable to conceive for over nine months.

Her first live pregnancy was associated with a flare of lupus at eight weeks, which settled by increasing her prednisolone dose to 8 mg od. She was already taking azathioprine 75 mg od and hydroxychloroquine 400 mg od. At 12 weeks, she complained of fatigue only. At 21 weeks, she was well, with no active lupus but later the pregnancy was complicated by worsening fatigue, joint pain, swelling and difficulty moving. She delivered a healthy baby at 33 weeks at another hospital, although it was monitored on SCBU for two weeks. Post partum her symptoms worsened and her steroids were increased to 15 mg od for one month, tapered down to 10mg maintenance dose. She continued with hydroxychloroquine and azathioprine at the same doses.

Two years later her lupus became active again and she was treated with rituximab and the azathioprine was stopped. Her anti-dsDNA antibody level was less than 10 IU/L and C3 level was 1.25 g/L prior to therapy and they remained within the normal range following therapy. She became pregnant for the fourth time 59 weeks after her first rituximab infusion.

Her B cells were undetectable six weeks after rituximab was given but 26 weeks later her B cell count had risen to 0.014 x 10^9/L and peaked at 0.023 x 10^9/L after another 16 weeks. Her B cell count did not change significantly after she conceived.

A malar rash appeared at the end of the first trimester (1 x BILAG B score). At 33 weeks following conception she complained of arthralgia and mild dyspnea (1 x BILAG B score), which improved after increasing the dose of prednisolone to 15 mg od. There were no changes in her anti-dsDNA antibody and C3 levels during the pregnancy. She delivered a girl weighing 2210 g at 35+5/40 by normal vaginal delivery. Ten weeks after she gave birth her B cell count fell to 0.012 x 10^9/L.

She had a partial collapse of the 8^{th} thoracic vertebra consistent with osteoporosis three months post-partum. She was started on risedronate and her prednisolone dose was reduced

but her arthralgia worsened and she was then retreated with azathioprine (1 x BILAG B score).

Patient 24 is a 40-year-old mixed race woman with a history of arthralgia, serositis, cutaneous vasculitis and digital arterial thrombosis. She was positive for RNP antibodies and was negative for lupus anticoagulant and anticardiolipin antibodies. She had had no previous pregnancies.

Her disease remained active despite treatment with methotrexate, azathioprine and cyclophosphamide and she was then treated with her first cycle of rituximab. Prednisolone 5mg od was given as maintenance treatment. Her anti-dsDNA antibody level was 89 IU/L and C3 level was 1.25 g/L prior to therapy. After treatment her anti-dsDNA antibody level immediately fell to less than 10 IU/L and her C3 level increased to a peak of 1.77 g/L after 145 weeks. She became pregnant for the first time 260 weeks after her first rituximab infusion.

Her B cells were undetectable three weeks after rituximab was given and remained deplete 17 weeks later. They rose to 0.02 x 10^9/L 38 weeks after treatment and remained at this level until she conceived when her B cell count rose to 0.059 x 10^9/L at 20 weeks before falling to 0.013 x 10^9/L at 39 weeks. Her lupus remained quiescent during the pregnancy apart from alopecia (1 x BILAG B score), which was noted at 38 weeks, and her anti-dsDNA antibody and C3 levels remained within the normal range. She delivered a healthy baby at 40 weeks by Caesarean section at another hospital. Her B cells steadily rose to 0.068 x 10^9/L 47 weeks after she gave birth but there was no change in her condition or serology.

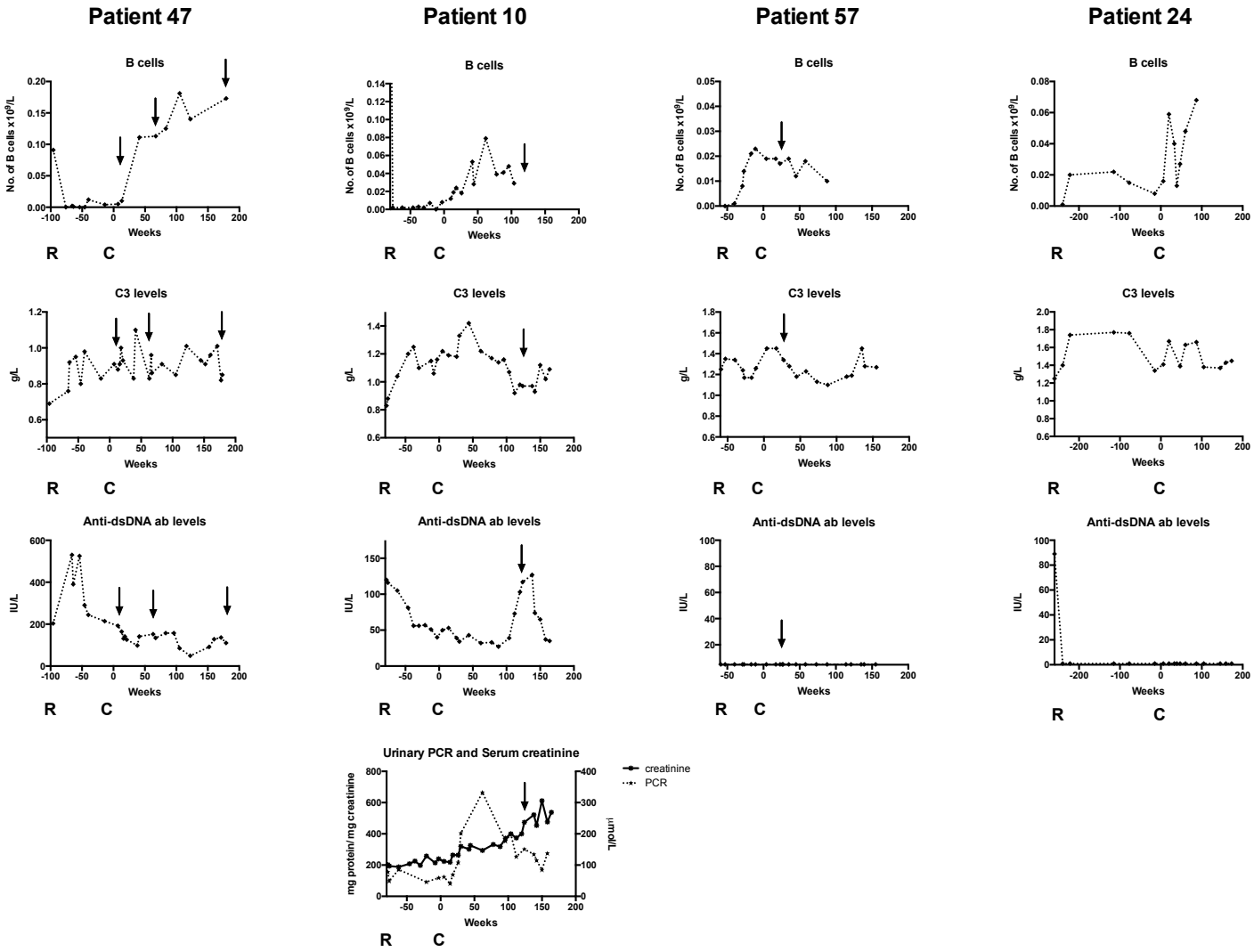


Figure 5.3.1 – Changes in B cell numbers, C3 levels and anti-dsDNA antibody levels during pregnancy in patients with SLE treated with BCDT

B cell numbers, C3 levels and anti-dsDNA antibody levels of patients 10, 24, 47 and 57, and urine protein/creatine ratio and serum creatinine level of patient 24, following rituximab therapy and then during pregnancy. The graphs start from the time of the first rituximab infusion (R). Week 0 represents the estimated time of conception (C). Arrows indicate disease flares.

5.3.3 Discussion

These four cases show that pregnancy might be an additional cause for B cell expansion and activation in some patients. During healthy pregnancy the immune system changes in order to prevent rejection of the semi-allogeneic foetus. In healthy pregnant mice sex steroids reduce B lymphopoiesis by inhibiting the IL-7 responsiveness of B cell precursors (479), possibly explaining an observed reduction in the numbers of B cell precursors and increase in the number of mature B cells (480). Early studies in healthy pregnant women showed that the concentrations of circulating immunoglobulin do not appear to change and immunization to tetanus toxoid results in a normal humoral response, suggesting that B cell function does not change (481). However, later studies have shown that pregnancy is associated with an increase in antibodies that protect the pregnancy, called assymetric antibodies because they have had an oligosaccharide group of the high mannose type added to one of the two Fab fragments during post-translational modification (482). These antibodies bind to paternal antigens but do not activate an immune effector function. A subset of transitional B cells that secrete IL-10, called regulatory B cells, might also be important for protecting the pregnancy. These regulatory B cells express the receptor for the human chorionic gonadotrophin hormone (hCG), which might encourage its expansion during the early stages of pregnancy when hCG levels rise (483). There appears, however, to be a lack of studies examining possible changes, both functional and phenotypic, in mature B cells during healthy pregnancy in humans.

The higher incidence of SLE in women and the observations that it is often worse during specific phases of the menstrual cycle, pregnancy or when patients take the oral contraceptive pill has implicated the female sex hormones in the disease pathogenesis, in particular oestrogen and prolactin. These two hormones have been shown to activate B cells and cause an increase in autoantibody production in several murine models (119, 484), although they appear to cause differences in the maturation of identical DNA reactive B cells with oestrogen causing the B cells to become marginal zone B cells and prolactin causing the B cells to

become follicular B cells (484). *In vitro* studies show that both hormones can induce autoantibody production by B cells from SLE patients when they are cultured with other mononuclear cells (485, 486), but studies comparing sex hormones with changes in B cell numbers and function during pregnancy in patients with SLE are lacking.

A study of patients with pre-eclampsia has intriguingly shown that the $CD5^+$ B cell subset, which produces autoantibodies against angiotensin type 1 receptor II and are found in the placenta of pre-eclamptic pregnancies, can be expanded by hCG (487). As hCG is elevated in the first few weeks of pregnancy this would be a possible candidate for the cause of B cell expansion that occurred shortly after conception in patients 10 and 47.

After pregnancy, when the pregnancy related hormones were no longer high, the B cell numbers were maintained within normal levels. The mechanism that sustains B cell numbers after repopulation is not known, but it is possibly induced by autostimulation, either by cytokine production or direct cell contact (280, 488, 489).

This study also shows that there are three distinct patterns of relapse following B cell repopulation after BCDT, with regards to changes in anti-dsDNA antibody levels and circulating B cell numbers.

1. Patient 47 went into remission even though her anti-dsDNA antibody levels did not fall but relapsed as soon as the B cells started to repopulate without a rise in her anti-dsDNA antibody levels. The low B cell count at the time of relapse was consistent with the observation made in chapter 5.1 that patients with high anti-dsDNA antibody levels relapse with low B cell numbers. This observation suggests that anti-dsDNA antibodies may cause disease by interacting with B cells either directly or indirectly.

2. Patient 10 went into remission when her B cells were depleted but after repopulation there was a two-year delay before her anti-dsDNA antibody levels rose with a large rise in her creatinine. During those two years she had a series of non-specific changes, including a rise in her blood pressure, headaches, proteinuria and a small

rise in her creatinine, which indicated a problem within her kidneys but could not be clearly attributed to active glomerulonephritis, as this would have required a renal biopsy, particularly in the absence of haematuria or rapidy deteriorating renal function. The source of her anti-dsDNA antibodies was not certain but as her only problems prior to the rise in anti-dsDNA antibody levels arose from her kidneys (indicated by the proteinuria, hypertension and creatinine rise), it is tempting to speculate that they may have arisen from B cells in her kidney.

3. Patient 57 did not have any anti-dsDNA antibodies in her serum and normal complement levels but still responded to BCDT. Her first symptom of this relapse was a malar rash, which is consistent with studies that show that the malar rash is an early symptom of SLE (110, 112). The absence of anti-dsDNA antibodies raises the possibility that the relapse was due to an antibody independent function of B cells. In the next chapter I will compare the skin histology of two patients with low anti-dsDNA antibody levels who had early/low-grade disease with malar rashes with the skin histology from three patients with high anti-dsDNA antibody levels who had chronic and/or multi-systemic disease with diffuse skin rashes.

Interestingly, in one murine model of SLE, oestrogen was shown to exacerbate immune complex mediated glomerulonephritis but ameliorate T cell mediated vasculitis (490). This might explain the lack of flare that was observed in patient 24 who was initially treated for a vasculitis but had no symptoms of active SLE and low anti-dsDNA antibody levels during the pregnancy even though the B cells had repopulated. Anti-dsDNA antibodies and low complement are associated with pregnancy-related complications in SLE (491), which supports the murine study that suggests that immune complex mediated disease is more susceptible to pregnancy-related flare.

This study shows that an antibody-independent function of B cells might also be an important factor in active disease. More research is needed to confirm this observation and to

understand which B cell subset is involved and how they might induce inflammation. In the next chapters, the possible mechanisms behind these three different disease patterns and the possible antibody-independent role of B cells in SLE will be examined.

5.4 Lymphocytic infiltrates in active skin lesions of patients before treatment with BCDT might differ according to anti-dsDNA antibody levels

5.4.1 Introduction

In chapter 5.1 it was shown that disease relapse was associated with different B cell numbers and phenotypes depending on whether patients had low or high anti-dsDNA antibody levels. Anti-dsDNA antibodies have been strongly associated with renal disease (187) but associations between anti-dsDNA antibodies and other manifestations of the disease are less clear (140). This chapter describes the histopathology from skin biopsies of active lesions from five patients before they had BCDT and compares the histology with their anti-dsDNA antibody levels.

5.4.2 Results

Patient 62 presented with a plaque and swelling on her left cheek, patient 78 had a chronic bilateral malar rash with acneform erythematous papules, patients 54 and G had sudden-onset diffuse erythema affecting their face, trunks and limbs and patient F had diffuse discrete macular and annular lesions on her face, trunk and arms. Biopsies were therefore taken from the malar region from patients 62 and 78 and from the arms from patients 54, F and G (Table 5.4.1).

Histological evidence of active lupus was seen in two of the biopsies, from patients 62 and 54. In the biopsy of patient 62 there was mild hyperkeratosis, occasional follicular plugging, mild atrophy of the epidermis and lymphocytic infiltrates in the epidermis, dermoepidermal junction, around the blood vessels and hair follicles consistent with chronic cutaneous lupus erythematosus. In the biopsy of patient 54 the epithelium showed hyperkeratosis. There was also spongiosis and basal cell vacuolation damage associated with lymphocyte exocytosis from the dermis and the dermis showed mild oedema with a lymphocytic infiltrate consistent with acute cutaneous lupus erythematosus. The biopsy of patient 78 showed features more consistent with ulceration. The biopsy of patient F the features were non-specific and the biopsy of patient G showed features consistent with 'burnt-out' interface dermatitis possibly due to previous lupus.

Patient 62 had an anti-dsDNA antibody level of 11 IU/L and normal complement level at the time that the biopsy was taken but the biopsy revealed a dense lymphocytic infiltration of the dermis. Patient 78 also had dense lymphocytic infiltrate associated with a mildly raised anti-dsDNA antibody level of 62 but normal complement level, whilst in patient 54, who had a mildly raised anti-dsDNA antibody level of 70 IU/L and reduced complement level of 0.6 g/dL, there was only scattered lymphocytic infiltration within the dermis and a perivascular lymphocytic infiltrate. In patients F and G who had the highest anti-dsDNA antibody levels (366 and 300 respectively) there were only perivascular lymphocytic infiltrates. Circulating lymphocyte numbers were at the lower limit of normal in patients 62 and 78 (1.58 and 1.56

respectively) but they fell after the biopsy was taken. The circulating lymphocyte numbers in patients 54, F and G were all significantly below normal.

Immunohistochemical stains for the lymphocyte markers CD3, CD4, CD8, CD20 and the monocyte marker CD14 were performed. The stains revealed that the infiltrates were only $CD3^+$ T cells in the biopsies of patients 62, 54, F and G. In the biopsy of patient 78 the lymphocytes were mostly $CD3^+$ T cells, of which 70% were $CD4^+$, but small aggregates of $CD19^+$ B cells could also be seen within the T cell infiltrates. A few $CD14^+$ monocytes were also seen in the biopsies of patients 78, F and G (data shown in chapter 5.7).

Direct immunofluorescence showed immune deposits consistent with cutaneous lupus in the biopsies of patients 62, 54, F and G. An immunofluorescent stain was not performed on the biopsy of patient 78.

Patients 62, F and G were treated with rituximab shortly after the biopsies were taken, which was followed by an improvement in the skin lesions and extracutaneous symptoms in those patients. Patient 78 was initially given topical tacrolimus in addition to regular low-dose oral prednisolone and hydroxychloroquine but six months later she developed a diffuse cutaneous vasculitis associated with a rise in her anti-dsDNA antibody level to 2686 IU/L, which then led to her being treated with rituximab. Patient 54 was initially treated with intravenous methylprednisolone and cyclophosphamide but she relapsed five months later and was then treated with rituximab.

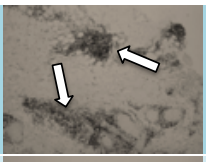
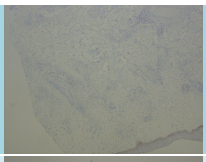
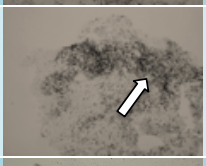
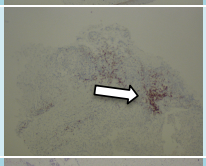
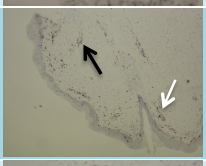
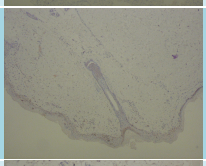
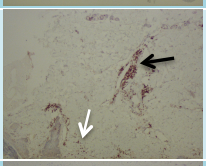
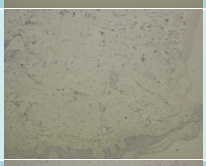
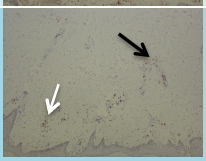
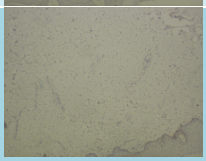
ID	CD3+ cells	CD19+ cells	IMF	Disease duration	Symptoms	ENA	Anti-dsDNA (IU/L)	C3 (g/dL)	Lymph ($x10^9$/L)
62			Granular deposits of IgM and C3 at the BMZ. In addition there is epidermal ANA staining.	7 months (2008)	Malar rash, fatigue and arthralgia	Ro+ La+	11	0.83	1.58
78			Data not available	11 years (1999)	Malar rash only. No constitutional symptoms (Digital vasculitis and fever after 9 months)	RNP+ Sm(+) Ro(+)	62 (2686 after 9 months)	1.07 (0.30 after 9 months)	1.56 (1.06 after 9 months)
54			Speckled BMZ staining with IgM, IgA and to a lesser extent with C3.	22 years (1985)	Diffuse desquamating rash, serositis and fever	Negative	70	0.60	0.27
F			Granular deposits of IgM, IgG, IgA and C3 at the BMZ.	7 years (2002)	Diffuse macular rash, alopecia, mouth ulcer, nephritis (class II), arthralgia, and fever	Ro+	366	0.39	0.49
G			Granular deposits of IgM, IgG, IgA at the BMZ and colloid bodies labelling with IgM, IgG and IgA in the papillary dermis and along the dermo-epidermal junction.	1 month (2010)	Diffuse desquamating rash, hair loss, thrombocytopaenia, arthralgia, weight loss and fever	Negative	300	0.38	0.89

Table 5.4.1 – Comparison between lymphocytic infiltrates in lupus skin lesions and anti-dsDNA antibody and C3 levels before BCDT

Immunohistochemical analysis of T ($CD3^+$) and B ($CD19^+$) lymphocytes in tissue biopsies of active skin lesions from 5 patients with SLE who were rituximab naïve (ID numbers 54, 62, 78, F and G). Original magnification: x40. Results from the direct immunofluorescence are also given with symptomatology, disease duration, ENA antibody seropositivity, anti-dsDNA antibody levels, C3 levels and lymphocyte numbers. Three types of lymphocytic infiltration are shown; interstitial aggregates (large white arrows), scanty interstitial infiltration (small white arrows) and perivascular aggregation (small black arrows). $CD3^+$ T cells were seen in all biopsies but $CD19^+$ B cells were only seen in the biopsy of patient 78.

5.4.3 Discussion

Skin lesions occur in 72-85% of patients with SLE (492). There are four types of lesions in SLE according to the Dusseldorf classification; acute cutaneous lupus erythematosus (ACLE), which includes a localized form (malar dermatitis) and a generalized form, subacute cutaneous lupus (SCLE), chronic cutaneous lupus erythematosus (CCLE), including discoid lesions, and intermittent cutaneous lupus erythematosus (ICLE).

Skin lesions in active lupus are classified histologically into early, fully developed, late or special manifestations (e.g. lupus erythematosus profundus) (126). The histological findings of early lupus erythematosus include sparse superficial perivascular lymphocytic infiltrates, similar to the infiltrates that were seen in the biopsy of patient 54. The histological findings of fully developed lupus erythematosus include dense perivascular and periadnexal lymphocytes in the papillary and reticular dermis, similar to the findings that were seen in the biopsy of patient 62. Therefore, histological chronicity is partly determined by the extent of lymphocytic infiltrates. However, this appears to contrast with the time since diagnosis and the anti-dsDNA antibody and C3 levels that were seen in the sera of patients 62 and 54, which suggested that patient 62 had early disease and patient 54 had chronic disease with an acute flare.

The role of autoantibodies in cutaneous lupus is unclear. Whilst immune deposits are often seen in active lesions, particularly at the dermoepidermal junction, they can also be seen in non-lesional skin (493). Ro antibodies, which were found in three of the patients, have been associated with photosensitivity and also the malar rash (494, 495), Sm and RNP antibodies, which were found in patient 78, have been associated with the malar rash in two studies (140, 494) and Ribosomal P antibodies have been associated with alopecia (140), but there is no clear explanation for how these antibodies might mediate skin inflammation.

Anti-dsDNA antibodies are often seen in patients with acute cutaneous LE lesions (496) and titres also correlate with the intensity of dermoepidermal junction immunofluorescence in the

lupus band test (493). One study has shown a possible association between anti-dsDNA antibodies and the malar rash, although this was seen in patients that also had Sm antibodies (495). Extracellular DNA has been seen in lesional skin in the basement membrane zone, vascular wall, and hair follicles (497), which could potentially complex with circulating anti-dsDNA antibodies.

The malar rash has been shown to be a common early manifestation of SLE (496) and a feature of ILE that predicts the development of complete SLE (110, 112). Lymphocytic infiltrates are a consistent finding in early discoid lupus (126) but immune deposits are not (493), suggesting that, in discoid lupus, lymphocytes migrate to the skin before immune complexes are deposited.

Whilst the numbers in this study are small, it is possible to suggest that there are at least four patterns of lymphocytic infiltration in the inflamed skin of patients with active disease; localized lesions with dense T cell infiltrates alone, dense T cell infiltrates with B cells, and diffuse lesions with perivascular infiltrates +/– scattered lymphocytic infiltrates. Importantly the presence of T cells in the skin precedes the appearance of anti-dsDNA antibodies in the serum, and also appears to be inversely related to the anti-dsDNA antibody levels and hypocomplementaemia.

It is intriguing that B cells are seen aggregating with T cells in the biopsy of patient 78 shortly before there was a sharp rise in her anti-dsDNA antibody levels and it is tempting to speculate that the cells were forming ELT, which produces plasma cells capable of producing the anti-dsDNA antibodies that then contributed to the more diffuse cutaneous vasculitis. This hypothesis is supported by three larger studies of renal biopsies that showed similar T and B cell infiltrates in conjunction with more severe immune-complex mediated pathology. The first study of seven renal biopsies showed $ICOS^+$ T cells in close contact with B cells and plasma cells in tissue that also stained for prominent IgG, IgA and IgM deposits (265). The presence of $ICOS^+$ T cells is significant because these cells have been shown to activate B

cells and enhance the production of anti-dsDNA antibodies (288). The second study of 68 renal biopsies showed features of higher order lymphoid structures composed of both B and T cells in the tubular interstitium that were strongly associated with immune complex deposition in the tubular basement membrane (152). There was evidence of intrarenal B cell clonal expansion and ongoing somatic hypermutation, which the authors concluded was driven by the coresident T cells. Sixty-five of the 68 biopsies showed features of either class III, IV or V glomerulonephritis but a correlation between the different lymphoid-like structures and the glomerular lesions was not performed. Nevertheless, they concluded that the deposition of immune complexes in glomeruli likely arises from a breach in systemic tolerance but that interstitial nephritis is associated with *in situ* tolerance diatheses. In other words they conclude that there are two separate immune complex-mediated inflammatory processes - one that starts in the SLOs and the other within the inflamed tissue.

The second study compared the B and T cell infiltrates in 192 renal biopsies (153) using a novel classification of renal histology according to the B cell infiltrates (498). The classification defines 5 distinct groups; grade 0 aggregates with T cell infiltrates alone, grade 1 aggregates with scattered T and B cells, grade 2 aggregates with cluster-like structures but no T- and B-cell zones, grade 3 aggregates with distinct T- and B-cell zones and grade 4 aggregates with the highest level of microanatomical organization with a central network of FDCs. They found that 38.5% of the biopsies were grade 0, 44.8% were grade 1, 8.9% were grade 2 and 7.8% were grade 3. There were no grade 4 type infiltrates. B cell aggregates were mostly seen in the tubulointerstitium rather than in the glomerulus. They found that the presence of B cells was associated with class IV nephritis, higher blood urea and creatinine levels and higher SLE Disease Activity Index (SLEDAI) scores but there were no differences between grades 1, 2 or 3. The presence of B cells was also associated with higher activity and chronicity indices. However, 24-hour urinary protein and anti-dsDNA antibody levels did not differ between the groups. The skin histology of patient 78 appeared similar to the grade 3 class, because the T and B cells were aggregating with distinct T and B cell zones. I did not

look for the presence of FDCs and, therefore, cannot be certain whether ELT was formed. However, two of the three studies that looked for the presence of FDCs found that they were seen in only a small number of specimens, suggesting that they might not be crucial for inducing B cell differentiation into antibody secreting plasma cells in non-lymphoid tissue.

These studies suggest that there is a specific inflammatory process within the non-lymphoid tissue, which is initiated by T cell infiltration, followed by B cells, then FDCs, which results in the formation of ELT. Plasma cells are then formed which produce autoantibodies that react to local antigen, form immune complexes and recruit other inflammatory cells (Figure 5.4.1). It could therefore be argued that the "fully developed" lesions are in fact part of an early localized inflammatory phase that precedes the systemic inflammation.

The formation of ELT is a normal part of the local immune response to infection (72) and it has been shown in mice that the first cells that infiltrate the tissue and initiate the formation of ELT are mature $CD3^{+}CD4^{+}$ T cells (499). However, ELT should disappear once the infection has been cleared. Therefore, the presence of large numbers of T cells in the tissue of patients with SLE suggests that there is a defect in their survival and retention within the tissue, which needs to be explained. It is also important to try to understand what induces their migration, why T cells should preferentially migrate to the face and kidneys, as seen in this study and the studies that were discussed above, and whether similar T cell infiltrates and ELT are seen in other organs that are typically affected in SLE (e.g. brain and joints). The recruitment and retention of T cells to specific tissue are likely to be controlled by the interaction of tissue-specific chemokine receptors and adhesion molecules expressed on the T cells with specific chemokines and integrins expressed by the endothelium and cells in the tissue. It is not known if the recognition of tissue antigen is important.

Given the good response of patient 62 to BCDT it is possible to hypothesize that B cells are inducing T cell migration. This hypothesis is supported by a study that genetically modified the B cells of MRL/*lpr* mice, which normally get a severe lupus-like disease associated with

antinuclear antibodies, so that they could not secrete immunoglobulin. The mice did not die as rapidly as the typical MRL/*lpr* mice but died earlier than mice that did not have any B cells (195). They did not get glomerulonephritis but did get an interstitial nephritis associated with a dense T cell infiltrate that looked similar to the T cell infiltrate seen in the skin biopsy of patient 62 and the T cell infiltrates in the renal histology studies that were discussed. Another study observed a reduction in the number of $CD3^+$ T cells in the renal biopsies in 50% of the patients who had been treated with BCDT (441). In multiple sclerosis and rheumatoid arthritis infiltrating T cells are also dependent on the presence of B cells (442, 500).

The next chapter will examine if and how B cells induce T cell activation and possible migration, particularly in patients with low levels of anti-dsDNA antibodies.

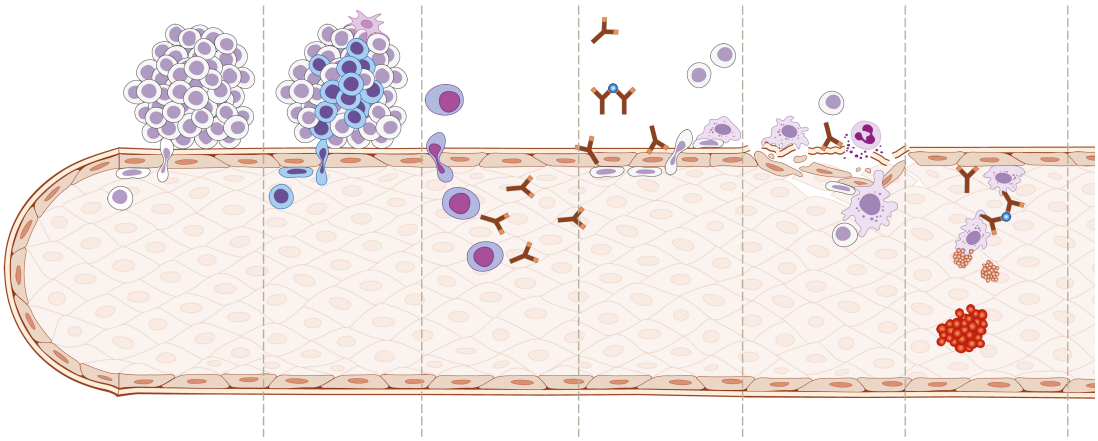


Figure 5.4.1 – T and B cells aggregate in the tissue in the early stages of inflammation
T cells are the first lymphocytes in the tissue. They then aggregate with B cells and follicular dendritic cells to form ectopic lymphoid tissue. With the appropriate stimuli in the ectopic lymphoid tissue B cells differentiate into antibody producing plasma cells, including plasma cells that produce anti-dsDNA antibodies.

T cell infiltration is the first stage in the formation of ectopic lymphoid tissue. The mechanisms that induce T cell infiltration are not fully understood but an animal study suggests that B cells might have an important role in the process (195). It was hypothesized that $IgD^{-}CD27^{-}$ B cells influence the migration of T cells into the non-lymphoid tissue.

5.5 HLA-DR expression by $CD4^+$ T cells is inversely related to $CD4^+$ T cell numbers in patients with low anti-dsDNA antibody levels and can be induced by BCR or TLR-9 stimulated B cells

5.5.1 Introduction

In chapter 5.1 it was observed that patients that relapsed with low anti-dsDNA antibody levels had high percentages of IgD^-CD27^- B cells. The function of these cells has not been established. In chapter 5.4 it was found that the two patients with low levels of anti-dsDNA antibodies had only localized cutaneous disease, with minimal systemic symptoms but the biopsies showed extensive T cell infiltration around the blood vessels and in the dermis. It was proposed that this might be the first step towards the development of ELT, which lead to the production of pathogenic autoantibodies and more diffuse cutaneous disease and systemic symptoms. It was hypothesized that IgD^-CD27^- B cells might induce the migration of T cells into the tissue.

Previous studies have shown that circulating $CD4^+$ T cells are dysregulated and activated. However, the functional role of activated $CD4^+$ T cells in SLE has been disputed. The most widely held view is that $CD4^+$ T cells induce autoantibody production, although it is not clear if this occurs in SLOs or the tissue. $CD4^+$ T cells also produce cytokines, which might activate other cells.

In this chapter I describe the changes in lymphocyte numbers after BCDT and examine whether they correlate with the expression of activation markers on $CD4^+$ T cells.

5.5.2 Results

B cell repopulation is associated with a fall in lymphocyte levels that coincides with or precedes clinical relapse

In the previous chapter I showed that the circulating lymphocyte numbers were on the lower limit of normal in the two patients with active cutaneous lupus and low anti-dsDNA antibody levels and continued to fall after the biopsies were taken but before the patients were given systemic therapy. Lymphocytes are mostly composed of $CD3^+$ T cells. It was therefore hypothesized that if B cells are contributing to T cell migration and if lymphopaenia is a consequence of T cell migration then lymphocyte numbers should fall when B cells repopulate after rituximab therapy.

The lymphocyte numbers were compared in patients with SLE who had been treated with rituximab at baseline, at the last period of B cell depletion, at the point at which B cells were first recorded to have repopulated above 0.01 x 10^9 cells/L and at 6 months after repopulation if they still had inactive disease or at relapse if the disease was active (Figure 5.5.1A). To examine if there were differences in the changes in lymphocyte numbers in patients with low or high anti-dsDNA antibody levels during remission or relapse patients were divided into a group with low anti-dsDNA antibody levels (<100 IU/L) that either remained in remission 6 months after repopulation (n = 13) or relapsed by 6 months after repopulation (n = 16), and a group that had high anti-dsDNA antibody levels that either remained in remission 6 months after repopulation (n = 8) or relapsed by 6 months after repopulation (n = 11).

In patients that relapsed with either low or high anti-dsDNA antibody levels there was a significant fall in lymphocyte numbers from baseline to depletion ($p = 0.01$ and $p = 0.04$ respectively, Wilcoxon matched-pairs signed rank test). This was possibly due to the depletion of B cells. From depletion to repopulation there was a significant rise in lymphocyte numbers in patients that relapsed with either low or high anti-dsDNA antibody levels ($p = 0.01$ and $p = 0.03$ respectively, Wilcoxon matched-pairs signed rank test), which was likely to

be due to the repopulation of B cells. However, in patients with low anti-dsDNA antibody levels that relapsed there was a fall in lymphocyte numbers between repopulation and relapse ($p = 0.03$, Wilcoxon matched-pairs signed rank test). A significant fall in lymphocyte numbers was not observed in the other groups. Given that B cell numbers are increasing during this period, the decrease in lymphocyte numbers that was seen in patients with low anti-dsDNA antibody levels that relapse was most likely to be due to a decrease in $CD3^+$ T cell numbers. In patients that remained in remission the only significant change in lymphocyte numbers that was observed was a rise between depletion and repopulation in the patients with high anti-dsDNA antibody levels ($p = 0.008$, Wilcoxon matched-pairs signed rank test).

A subanalysis showed that lymphopaenia at baseline is most likely to be due to low T cell numbers, particularly the $CD4^+$ subset, as T cell numbers were significantly lower in patients with active disease before BCDT ($n = 9$) compared to healthy controls ($n = 34$), with the greatest statistical significance in the $CD4^+$ subset ($p = 0.0005$ for $CD3^+$, $p = 0.003$ for $CD4^+$ and $p = 0.02$ for $CD8^+$ subsets, Mann-Whitney U tests). B cell numbers were not significantly lower in patients with active disease before BCDT (Figure 5.5.1B). However, both B and T cell numbers were significantly lower in patients in remission who have not been treated with rituximab ($n = 7$) compared to healthy controls ($p = 0.0007$ for B cells, $p = 0.006$ for $CD3^+$, $p = 0.006$ for $CD4^+$ and $p = 0.04$ for $CD8^+$ subsets Mann-Whitney U test). B cell numbers were also significantly lower in patients in remission compared to patients with active disease ($p = 0.04$, Mann-Whitney U test), which supports evidence that they are an important factor in active disease and might be causing the fall in T cell numbers, either directly or indirectly. The low T cell numbers in patients who are in long-term remission might support the observation that relapse is associated with a decrease in T cell numbers rather than the absolute number that is present in the circulation, which is further illustrated in the case studies below.

Case studies of patients with low levels of anti-dsDNA antibody levels at the time of B cell repopulation showing the rise in B cell numbers is first followed by a fall in lymphocyte numbers and then by a rise in anti-dsDNA antibody levels

The six cases shown in Figure 5.5.2 suggest a relationship between the repopulation of B cells with the subsequent fall in the lymphocyte numbers followed by a rise in anti-dsDNA antibody levels and flare of the disease in some patients. All six patients had anti-dsDNA antibody levels below 50 IU/L at the time of B cell repopulation. Patients 29, 39 and 11 had high percentages of Trm cells and patients 54, 51 and 61 had high percentages of Tem but low percentages of Trm cells. Trm cells have previously been shown to positively correlate with the percentage of $IgD^{-}CD27^{-}$ B cells, which were high during relapse in patients 29, 39 and 11. The percentages of $IgD^{-}CD27^{-}$ B cells in patients 51 and 61 were not available at the time of clinical relapse. The $CD4^{+}$HLA-DR^{+} T cells of the patients also had high expression of CD49d.

Patient 29 relapsed with arthralgia shortly after her B cells repopulated at week 34. Her lymphocyte levels fell from 1.89 to 0.76 x 10^{9} cells/L at week 44 but her anti-dsDNA antibody levels remained below 10 IU/L. Retreatment with rituximab improved her symptoms but the problems recurred each time her B cells repopulated, again coinciding with a fall in her lymphocytes from 1.47 to 0.75 x 10^{9} cells/L.

Patient 39 relapsed with arthritis shortly after her B cells repopulated at week 48, which coincided with a large fall in her lymphocyte levels from 4.09 to 2.79 x 10^{9} cells/L and a small rise in her anti-dsDNA antibody levels from 42 to 69 IU/L. She was retreated with rituximab and the B cells repopulated at 86 weeks, coinciding with a fall in her lymphocyte levels from 4.22 to 2.54 x 10^{9} cells/L and another rise in her anti-dsDNA antibody level from 28 to 66 IU/L.

The B cells of patient 11 repopulated at week 36. Her lymphocytes started to fall at week 64 from 1.92 to 1.56 x 10^{9} cells/L. She initially relapsed with a malar rash at week 74. Her rash

and lymphocyte level improved with a course of oral prednisolone but as the dose was reduced her lymphocyte level fell again to 0.34 x 10^9 cells/L and remained low, although there was not a recurrence of her rash. At week 290 she developed a nephrotic-like syndrome with an increase in her urinary protein levels, hypoalbuminaemia and peripheral oedema. She also developed memory loss, which was attributed to cerebral lupus. There had been a small but steady rise in her anti-dsDNA antibody level from less than 10 at B cell repopulation to 99 IU/L at week 308 when she had her second cycle of BCDT.

Patient 54 relapsed with serositis when her B cells repopulated at week 30, which was followed by a fall in her lymphocyte count from 0.93 to 0.56 x 10^9/L. She was initially treated with intramuscular steroids but her pain worsened and she developed a skin rash at week 71 after her lymphocyte count fell again from 1.11 to 0.44 x 10^9 cells/L and her anti-dsDNA antibody level had risen from 72 to 96 IU/L. At baseline she had had a skin biopsy, which showed a scattered lymphocytic infiltration within the dermis and a perivascular lymphocytic infiltrate that was only composed of $CD3^+$ T cells, shown in chapter 5.4, supporting the hypothesis that the fall in the lymphocyte count in the blood is due to sequestration of T cells into the tissue.

The B cells of patient 51 repopulated at week 37. Her lymphocyte count did not start falling until week 90, when her B cell count peaked at 0.114 x 10^9 cells/L. Her lymphocyte number fell from 2.12 to 0.66 x 10^9/L steadily over 120 weeks. Her anti-dsDNA antibody level only started rising at week 160 when it rose from less than 10 to 51 IU/L at week 196 when she developed a cutaneous vasculitis, abdominal pain and dyspnea.

The B cells of patient 61 repopulated at week 23. Her lymphocyte count did not start falling until week 62 when her B cell count peaked at 0.131 x 10^9 cells/L. Her lymphocyte number fell from 0.99 to 0.2 x 10^9 cells/L. Her anti-dsDNA antibody levels started rising at week 94 from 23 to 7472 IU/L when she developed a cutaneous vasculitis.

It has been suggested that lymphopaenia might be due to the lymphocytotoxic effects of anti-dsDNA antibodies (375), but the results of patients 29, 51 and 61 show that the fall in lymphocyte numbers is unlikely to be due to anti-dsDNA antibodies as the anti-dsDNA antibodies were either not detectable (patient 29) or appeared after the lymphocyte numbers fell. It is more likely that the falling lymphocyte numbers reflects a process of T cell migration and tissue infiltration that leads to the production of anti-dsDNA antibodies.

Following BCDT HLA-DR expression by $CD4^+$ T cells falls but only in patients with low anti-dsDNA antibody levels

It was hypothesized that B cells induce changes in $CD4^+$ T cells to induce their migration into the tissue. If disease is characterized by T cell infiltration and a fall in circulating lymphocyte numbers in patients with low anti-dsDNA antibody levels then the changes in $CD4^+$ T cells should be most noticeable in this group of patients. Studies have previously reported that the T cell expression of the activation markers, CD40L, CD69, HLA-DR and ICOS fall after BCDT in patients with SLE (290, 439, 501). The expression of the activation markers, CD69 and HLA-DR by $CD4^+$ T cells from patients were therefore compared before and after BCDT to confirm the results that were found in these studies and to determine whether changes in T cell activation markers differ in patients according to whether they had either low or high anti-dsDNA antibody levels (Figure 5.5.3).

Patients with low anti-dsDNA antibody levels did not have significantly higher percentages of HLA-DR^+ $CD4^+$ T cells at baseline (n = 15) compared to healthy controls (n = 24) or with patients with inactive disease who had low anti-dsDNA antibody levels (n = 13). The expression of HLA-DR was lower than baseline at 6 weeks after BCDT (n =15) ($p = 0.02$, Wilcoxon matched-pairs signed rank test, two tailed) and at 16 weeks (n = 14) ($p = 0.05$, Wilcoxon matched-pairs signed rank test, two tailed; $p < 0.03$, Wilcoxon matched-pairs signed rank test, one tailed). The absolute numbers of $CD4^+$HLA-DR^+ T cells at baseline were also similar to healthy controls and patients with inactive disease. The numbers of $CD4^+$HLA-DR^+ T cells was lower than baseline 6 weeks after BCDT ($p = 0.02$, Wilcoxon matched-pairs

signed rank test, two tailed) and at 16 weeks (p = 0.04, Wilcoxon matched-pairs signed rank test, two tailed) and increased when B cells repopulated compared to week 6 after BCDT (p = 0.03, Wilcoxon matched-pairs signed rank test, two tailed).

Patients with high anti-dsDNA antibody levels at baseline (n = 13) had similar percentages of HLA-DR$^+$ CD4$^+$ T cells compared to healthy controls (n = 24) and patients with inactive disease (n = 5) with high anti-dsDNA antibody levels. The expression of HLA-DR did not change after BCDT at week 6 (n = 13), week 16 (n = 8) or at repopulation (n = 7). The absolute numbers of CD4$^+$HLA-DR$^+$ T cells also did not differ significantly between patients with active disease at baseline and healthy controls and patients with inactive disease and did not change significantly after BCDT.

In patients with active disease at baseline the percentage of CD69$^+$ CD4$^+$ T cells was higher than in healthy controls in the groups with low anti-dsDNA antibody levels and the group with high levels (both $p < 0.001$, Mann-Whitney U test, two-sided). There was no difference seen between patients with active disease at baseline and patients with inactive disease and it did not change after BCDT in either the group with low anti-dsDNA antibody levels or the group with high levels. The absolute numbers of CD4$^+$CD69$^+$ T cells were not different between the healthy controls, the patients with active disease at baseline and the patients with inactive disease in either the group with low anti-dsDNA antibody levels or the group with high levels.

Expression of HLA-DR by CD4$^+$ T cells inversely correlates with absolute CD4$^+$ T cell numbers after B cells repopulate in patients with low anti-dsDNA antibody levels

A study has shown that during active SLE a fall in CD4$^+$ T cell numbers coincides with a rise in the percentage of CD4$^+$ T cells expressing HLA-DR and an increase in activated B cells (502). It was therefore hypothesized that there would be an inverse relationship between CD4$^+$ T cell numbers and HLA-DR expression by CD4$^+$ T cells, which would be dependent on the presence of B cells. CD4$^+$ T cell numbers were plotted against the percentages of

HLA-DR expressing $CD4^+$ T cells from patients treated with BCDT at least six months previously and then subanalysed according to whether B cells had repopulated to high numbers (>0.11 x 10^9 cells/L, n = 15), as defined in results chapter 5.1.2, low numbers (0.01 to 0.11 x 10^9 cells/L, n = 28) or were still deplete of B cells (<0.01 x 10^9 cells/L, n = 10).

There was a moderate inverse correlation between the absolute $CD4^+$ T cell numbers and the percentages of $CD4^+$ T cells expressing HLA-DR ($r_s = –0.47$, p = 0.0004) and a weak inverse correlation between the absolute $CD4^+$ T cell numbers and the percentages of $CD4^+$ T cells expressing CD69 ($r_s = –0.39$, p = 0.004) (Figure 5.5.4A).

The inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing HLA-DR was strong in patients with high B cell numbers ($r_s = –0.64$, p = 0.01) and moderate in patients with low B cell numbers ($r_s = –0.48$, p = 0.01). The inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing HLA-DR was not significant in patients who were still deplete of B cells ($r_s = –0.57$, p = 0.09). The inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing CD69 was strong in patients with high B cell numbers ($r_s = –0.63$, p = 0.01), but there was no significant correlation in patients with low B cell numbers or who were still deplete of B cells ($r_s = –0.28$, p = 0.14 and $r_s = –0.38$, p = 0.28 respectively) (Figure 5.5.4B).

It was shown in chapter 5.1.2 that relapse in patients with low anti-dsDNA antibody levels was associated with attainment of B cell numbers >0.11 x 10^9 cells/l. Amongst the 15 patients that had high B cell numbers two patients had an anti-dsDNA antibody level above 100 IU/L. When these patients were excluded the inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing HLA-DR became stronger ($r_s = –0.77$, p = 0.003). The inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing CD69 also became stronger ($r_s = –0.73$, p = 0.007) (Figure 5.5.5).

There were 16 patients in the group who had active disease. Three of the patients were still B cell deplete. The inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing HLA-DR was strong in the 13 patients that had active disease with B cells ($r_s = –0.68$, $p = 0.01$) but weak in the 30 patients in remission with B cells ($r_s = –0.39$, $p = 0.03$) (Figure 5.5.6A). Three of the 13 patients had anti-dsDNA antibody levels above 100 IU/L. When only the 10 patients with low anti-dsDNA antibody levels were included in the analysis the inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing HLA-DR again increased further ($r_s = –0.76$, $p = 0.01$) (Figure 5.5.6B). The inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing CD69 was moderate in the 13 patients that had active disease with B cells ($r_s = –0.57$, $p = 0.04$) but not significant in the 30 patients in remission with B cells ($r_s = –0.35$, $p = 0.06$) (Figure 5.5.6A). However, when the three patients with high anti-dsDNA antibody levels were excluded from the group with active disease the inverse correlation between the $CD4^+$ T cell numbers and the percentage of $CD4^+$ T cells expressing CD69 was no longer significant ($r_s = –0.52$, $p = 0.13$) (Figure 5.5.6B).

CD49d expression by $CD4^+HLA\text{-}DR^+$ T cells is higher in patients with active disease following B cell repopulation

Studies have shown that $CD3^+$ T cells in inflamed tissue in patients with SLE and other autoimmune rheumatic diseases, express HLA-DR and adhesion molecules (503-505), suggesting that $CD4^+HLA\text{-}DR^+$ T cells are inclined to migrate from the blood to the tissue. The adhesion molecules LFA-1 and VLA-4 are expressed at high levels on the $CD4^+$ T cells of patients with SLE, although high VLA-4 expression was only seen in patients that developed vasculitis (164). Consistent with these studies, I found that the expression of CD49d, the alpha-4 subunit of VLA-4, was higher on the $CD4^+HLA\text{-}DR^+$ T cells from patients with active disease (n = 13) compared to patients that were in remission after B cells had repopulated following BCDT (n = 25) ($p < 0.05$, Mann-Whitney U test) (Figure 5.5.7). However, none of the patients that relapsed had vasculitis. It was also observed that in

patients with active disease, $CD4^+HLA\text{-}DR^+$ T cells expressed higher levels of CD49d than $CD4^+HLA\text{-}DR^-$ T cells ($p = 0.003$, Wilcoxon matched-pairs signed rank test), suggesting that $CD4^+HLA\text{-}DR^+$ T cells are more likely to adhere to the vascular endothelium.

HLA-DR expression by $CD4^+$ T cells positively correlates with the percentage of IgD^-CD27^- B cells before and after BCDT

In chapter 5.1 it was shown that clinical relapse with low anti-dsDNA antibody levels following BCDT was associated with high percentages of IgD^-CD27^- B cells. As the fall in HLA-DR expression by $CD4^+$ T cells following BCDT was only seen in patients with low anti-dsDNA antibody levels it was decided to test whether HLA-DR expression by $CD4^+$ T cells correlated with IgD^-CD27^- B cells. Comparison of the expression of HLA-DR by $CD4^+$ T cells with memory B cell subsets in healthy individuals (n = 14) and SLE patients who had not been treated with BCDT (n = 41) (Figure 5.5.8A) showed that there was a moderate positive correlation between the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells and the percentage of IgD^-CD27^- B cells ($r_p = 0.48$, $p = 0.002$). There was also a moderate negative correlation between the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells and the percentage of IgD^+CD27^+ B cells ($r_p = –0.42$, $p = 0.006$). There were no significant correlations between the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells and the percentage of IgD^-CD27^{int} B cells or IgD^-CD27^{hi} plasmablasts.

A comparison of the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells and memory B cell subsets in patients treated with BCDT following B cell repopulation (n = 32) showed there was again a modest positive correlation between the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells and the percentage of IgD^-CD27^- B cells ($r_p = 0.34$, $p = 0.05$) (Figure 5.5.8B). However, in contrast to the SLE patients and healthy individuals who had not been treated with rituximab there was not a significant correlation between the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells and the percentage of IgD^+CD27^+ B cells. There were also no significant correlations between the percentage of $HLA\text{-}DR^+$ $CD4^+$ T cells and the percentage of IgD^-CD27^{int} B cells or the percentage of IgD^-CD27^{hi} plasmablasts.

During relapse the fall in $CD4^+$ T cell numbers is related to the expression of HLA-DR but during remission the rise in $CD4^+$ T cell numbers is independent of HLA-DR expression

Figure 5.5.9 shows the changes in HLA-DR expression by $CD4^+$ T cells with changes in $CD4^+$ T cell numbers at different stages after BCDT; the first stage shows the changes in the first six months after BCDT, the second stage shows the changes during a later period after BCDT with prolonged B cell depletion and the third stage shows the changes when B cells repopulate. Patient 11 shows that HLA-DR expression falls immediately after BCDT but $CD4^+$ T cell numbers remain low for the first 22 weeks. Patient 10 shows that in a patient who is still deplete of B cells between 30 and 73 weeks there is an increase in $CD4^+$ T cell numbers but this is not related to HLA-DR expression by $CD4^+$ T cells. Patient 56 shows that as B cells repopulate the $CD4^+$ T cell numbers decrease as HLA-DR expression by the $CD4^+$ T cells increase.

B cells stimulated with either hypomethylated DNA or a BCR stimulant induce HLA-DR expression via LFA-2

EBV-transformed B cells can transfer HLA-DR to T cells via cognate interactions (506). Increased B cell activity in SLE has been attributed to abnormal signaling via the BCR (507, 508) and TLR-9 (509). It was therefore decided to test whether BCR or TLR-9 stimulation of PBMCs from healthy controls (n = 5) and SLE patients (n = 5) for 96 hours could induce B cells to activate $CD4^+$ T cells. $(Fab')_2$ anti-human IgM was used to stimulate the BCR and CpG 2006, which is hypomethylated DNA (198), was used to stimulate TLR-9. There was an increase in HLA-DR expression by $CD4^+$ T cells when PBMCs from the healthy controls and SLE patients were stimulated with either $(Fab')_2$ anti-human IgM ($p = 0.02$ for healthy controls and $p = 0.04$ for SLE patients, paired t test) or CpG 2006 ($p = 0.002$ for healthy controls and $p = 0.009$ for SLE patients, paired t test). The B cells were depleted from the PBMCs to determine whether they were involved in the $(Fab')_2$ anti-human IgM or CpG 2006 induced expression of HLA-DR by $CD4^+$ T cells. The depletion of B cells from the PBMCs

of the healthy controls and SLE patients resulted in reduced expression of HLA-DR by $CD4^+$ T cells of unstimulated PBMCs from the SLE patients ($p = 0.02$, paired t test), but not of unstimulated PBMCs from the healthy controls, and PBMCs stimulated with $(Fab')_2$ anti-human IgM ($p = 0.04$ for healthy controls and $p = 0.02$ for SLE patients, paired t test) or CpG 2006 ($p = 0.01$ for both healthy controls and SLE patients, paired t test) (Figure 5.5.10A). The expression of HLA-DR by $CD4^+$ T cells did not differ between healthy controls and SLE, possibly because the PBMCs were taken from SLE patients without active disease at the time of the study. By contrast, stimulation of PBMCs with $(Fab')_2$ anti-human IgM resulted in a significant increase in the expression of CD69 by $CD4^+$ T cells from PBMCs of the healthy controls ($p = 0.04$, paired t test) but not from PBMCs of patients with SLE. Stimulation of PBMCs with CpG 2006 did not result in a significant change in CD69 expression by $CD4^+$ T cells from healthy controls or SLE patients. The depletion of B cells from PBMCs resulted in a surprising rise in CD69 expression by $CD4^+$ T cells from unstimulated PBMCs of the healthy individuals ($p = 0.03$, paired t test) but not of the SLE patients. There were no other changes in CD69 expression of $CD4^+$ T cells from PBMCs depleted of B cells.

To test whether the increased expression of HLA-DR by $CD4^+$ T cells requires cognate interactions between $CD4^+$ T cells and APCs, including B cells, $(Fab')_2$ anti-human IgM stimulated PBMCs were co-cultured with an antibody that blocks LFA-2 (CD2), which is a cell adhesion molecule found on T cells that binds to LFA-3 on APCs (510). Stimulation of PBMCs with $(Fab')_2$ anti-human IgM resulted in an increase in the expression of HLA-DR by $CD4^+$ T cells from PBMCs of healthy controls ($p = 0.009$, paired t test) and SLE patients ($p = 0.03$, paired t test). Blockade of LFA-2 resulted in reduced HLA-DR expression by $CD4^+$ T cells from unstimulated PBMCs of healthy controls ($p = 0.001$, paired t test) and SLE patients ($p = 0.008$, paired t test) and from $(Fab')_2$ anti-human IgM stimulated PBMCs of healthy controls ($p = 0.003$, paired t test) and SLE patients ($p = 0.02$, paired t test) (Figure 5.5.10B). There was a small but significant increase in HLA-DR expression by $CD4^+$ T cells of PBMCs stimulated with $(Fab')_2$ anti-human IgM and co-cultured with anti-CD2 compared to $CD4^+$ T

cells of PBMCs stimulated with $(Fab')_2$ anti-human IgM without anti-CD2 ($p < 0.05$ for healthy controls and $p = 0.01$ for SLE patients, paired t test), suggesting that factors other than LFA-2 are also involved in $(Fab')_2$ anti-human IgM induced HLA-DR expression by $CD4^+$ T cells.

DNA antibody rich serum combined with plasmid DNA can increase CD69 but not HLA-DR expression by $CD4^+$ T cells in healthy PBMCs

Patient 47, who was previously discussed in chapter 5.3, had high anti-dsDNA antibody levels at the time that her B cells started to repopulate and experienced 3 clinical relapses that were not associated with a substantial rise in her anti-dsDNA antibody levels or a fall in C3 levels. However, her relapses were associated with a sharp fall in lymphocyte numbers (Figure 5.5.11A).

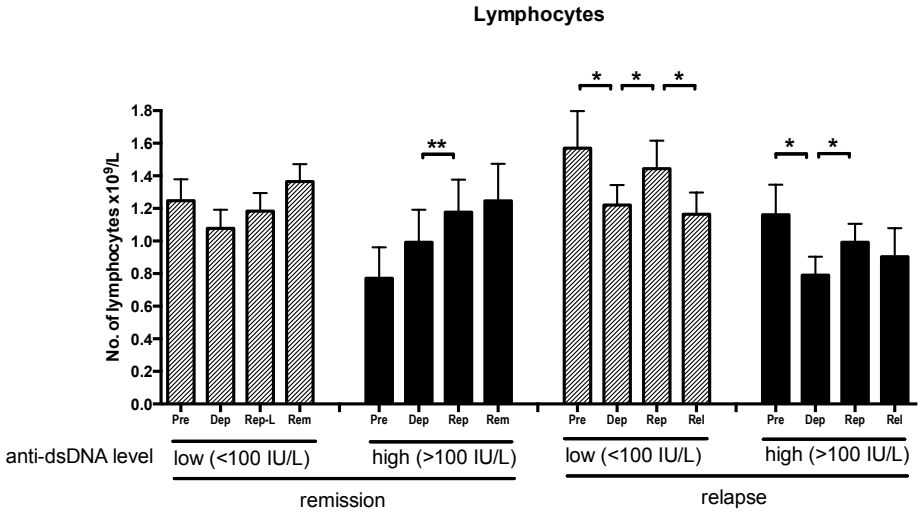
It was previously shown that hypomethylated DNA can induce HLA-DR expression by $CD4^+$ T cells when cultured with B cells. It was therefore hypothesized that anti-dsDNA antibodies induce HLA-DR expression by $CD4^+$ T cells. PBMCs from healthy individuals (n = 6) were therefore cultured in heat-inactivated sera from patients with high anti-dsDNA antibody levels (>100 IU/L) with CpG or plasmid DNA. The results show that the addition of CpG 2006 to the healthy and SLE serum resulted in an increase in HLA-DR expression by $CD4^+$ T cells ($p = 0.03$, Wilcoxon matched-pairs signed rank test) in contrast with the previous experiment, but was not affected by the SLE serum (Figure 5.5.11B). The addition of plasmid DNA to serum from SLE 2 also resulted in an increase in HLA-DR expression ($p = 0.03$, Wilcoxon matched-pairs signed rank test), but it was not significantly higher than the HLA-DR expression by $CD4^+$ T cells from the PBMCs cultured with healthy serum and plasmid DNA.

The expression of CD69 is upregulated by Type 1 IFNs (511), which are produced at higher levels when PBMCs are cultured with anti-dsDNA antibody rich serum and plasmid DNA (181). It was therefore hypothesized that combining anti-dsDNA antibodies with

unmethylated DNA might induce CD69 expression by $CD4^+$ T cells when added to PBMCs. In contrast to the previous experiment, the addition of CpG resulted in an increase in CD69 expression by $CD4^+$ T cells ($p = 0.03$, Wilcoxon matched-pairs signed rank test) but it was not affected by serum containing anti-dsDNA antibodies. Plasmid DNA also resulted in an increase in CD69 expression by $CD4^+$ T cells but it was only statistically significant when it was added to the serum from patients 1 and 2 ($p = 0.03$, Wilcoxon matched-pairs signed rank test) and it was significantly higher in the PBMCs that were cultured in the serum from patient 2 with plasmid DNA compared to the PBMCs that were cultured in healthy serum with plasmid DNA ($p = 0.03$, Wilcoxon matched-pairs signed rank test) (Figure 5.5.11B).

This experiment shows that B cells stimulated with CpG or via the BCR can induce HLA-DR expression by $CD4^+$ T cells but stimulation of B cells with immune complexes bound to DNA did not have any effect on HLA-DR expression by $CD4^+$ T cells, although it did increase CD69 expression.

A



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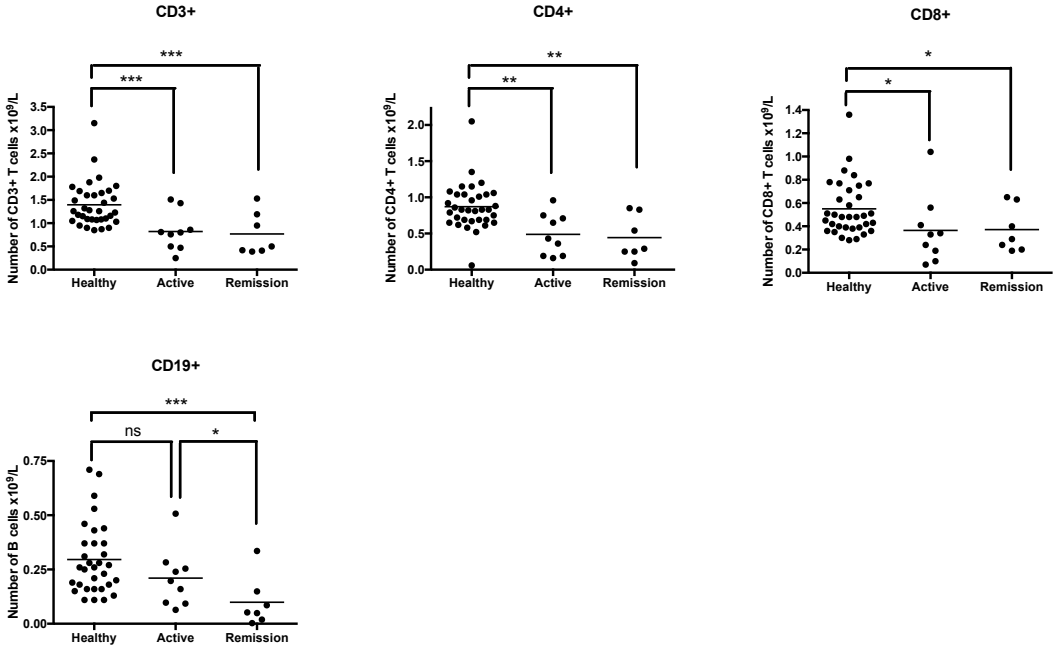


Figure 5.5.1 – Changes in lymphocyte numbers following BCDT in relapsing patients compared with those that remain in remission

(**A**) Lymphocyte numbers are shown at baseline (Pre), during B cell depletion (Dep), at B cell repopulation (Rep) and at remission (6 months post repopulation; Rem) or at clinical relapse in patients (Rel) who had either low anti-dsDNA antibody levels [low (<100 IU/L); n = 13 (remission), n = 16 (relapse)] and high anti-dsDNA antibody levels [high (>100 IU/L); n = 8 (remission), n = 11 (relapse)]. Columns indicate the means; bars indicate the S.E.M. Differences between the groups were analysed by Wilcoxon matched-pairs signed rank test. * $p < 0.05$, ** $p < 0.01$. (**B**) Comparison of T and B cell numbers between healthy controls [Healthy; n = 34], SLE patients with active disease at baseline [Active; n = 9] and rituximab naïve SLE patients in remission [Remission; n = 7]. Data are shown as dot plots with the mean represented by a horizontal bar. Differences between the groups were analysed by the Mann-Whitney U test. NS = $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

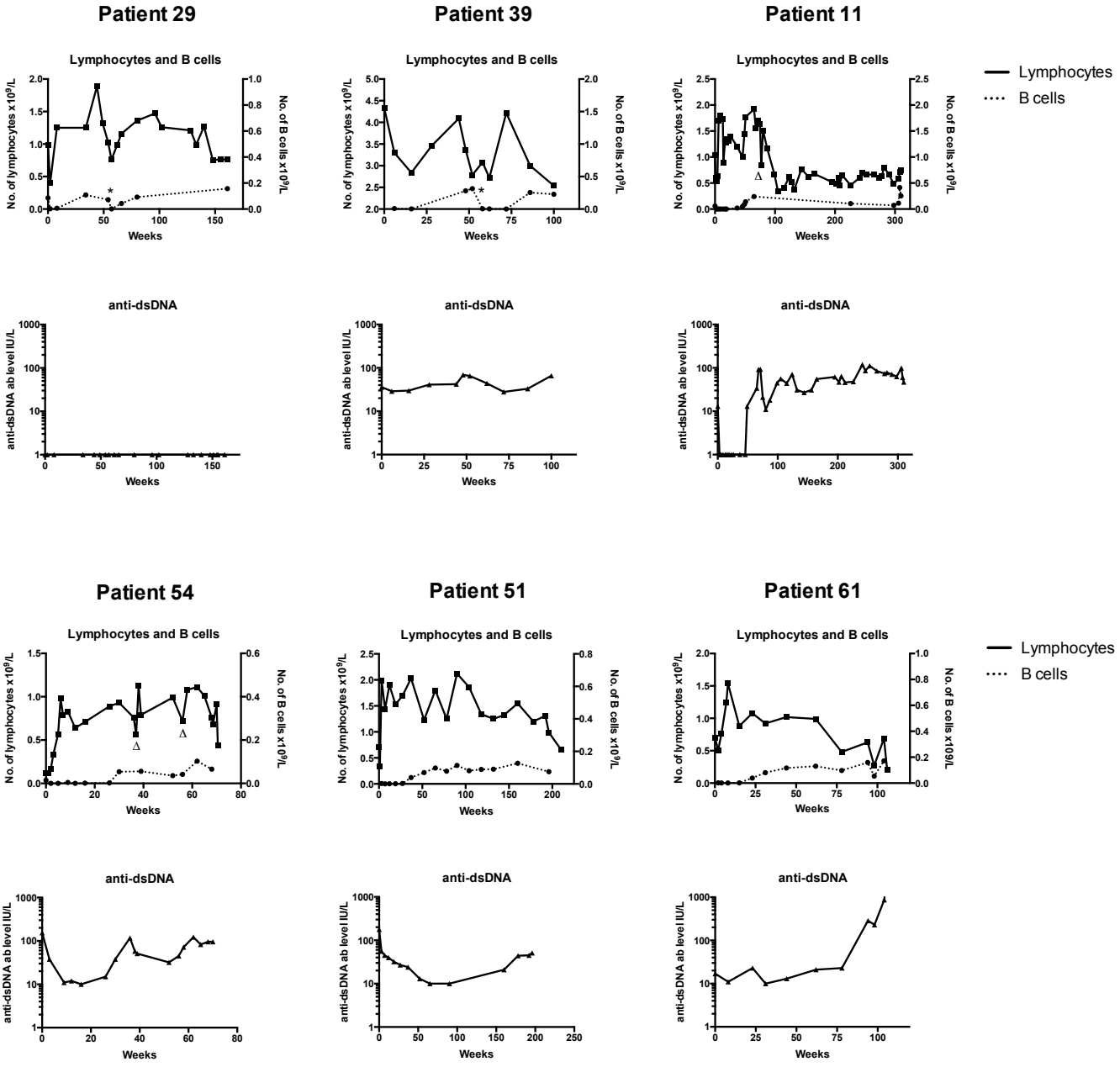


Figure 5.5.2 – Changes in lymphocyte numbers, B cell numbers and anti-dsDNA antibody levels following BCDT

Illustrations of the parallel changes in lymphocyte and B cell numbers and anti-dsDNA antibody levels following BCDT in six patients (ID numbers 11, 29, 39, 51, 54 and 61). The first cycle of therapy starts at week 0. An asterisk (*) is shown when BCDT is repeated (patients 29 and 39). Lymphocyte numbers (left Y-axis) and B cell numbers (right Y-axis) are both shown using a linear scale. A triangle (Δ) is shown when intramuscular methylprednisolone or a reducing course of high dose oral prednisolone was given for a flare of disease (patients 11 and 54). Anti-dsDNA antibody levels are shown using a logarithmic scale.

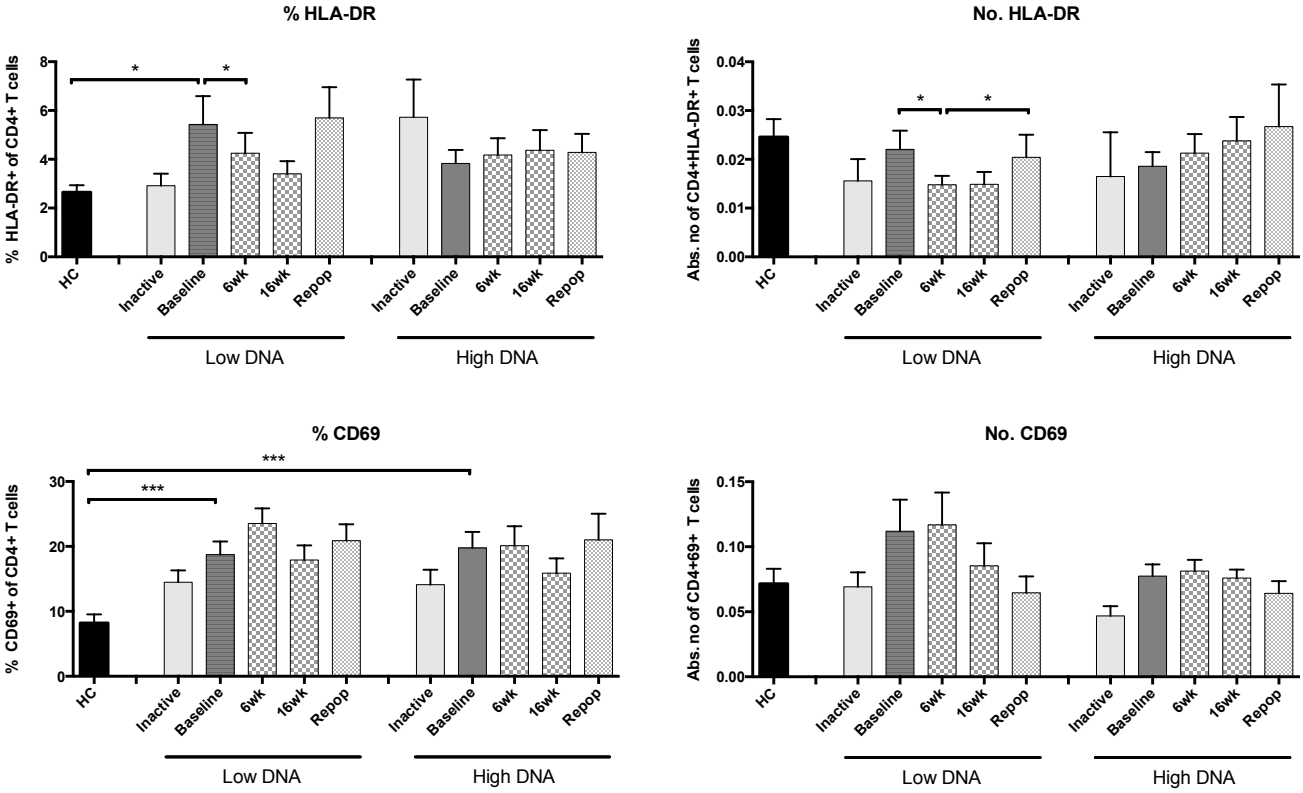


Figure 5.5.3 – Changes in the percentages and absolute numbers of $CD4^+$ T cells expressing the activation markers, HLA-DR and CD69, following BCDT

$CD4^+$ T cells from patients with SLE were analysed for changes in the percentages and absolute numbers of cells expressing HLA-DR and CD69 before and after BCDT. Healthy controls [HC; n = 24], SLE patients with low anti-dsDNA antibody levels (Low DNA (<100 IU/L)) with inactive disease [Inactive; n = 13], with active disease pre-BCDT [Baseline; n = 15] at weeks 6 [6wk; n = 15] and 16 [16wk; n = 14] after BCDT and/or after B cell repopulation (B cell numbers >0.01 x 10^9/L) [Repop; n=11] and SLE patients with high anti-dsDNA antibody levels (High DNA (>100 IU/L)) with inactive disease [Inactive; n = 5], with active disease pre-BCDT [Baseline; n = 13] at weeks 6 [6wk; n = 13] and 16 [16wk; n = 8] after BCDT and/or after B cell repopulation [Repop; n = 7]. Columns indicate the means; bars indicate the S.E.M. Differences between groups were analysed by Mann-Whitney U test for non-paired data or the Wilcoxon matched-pairs signed rank test for paired data. * $p < 0.05$, *** $p < 0.001$.

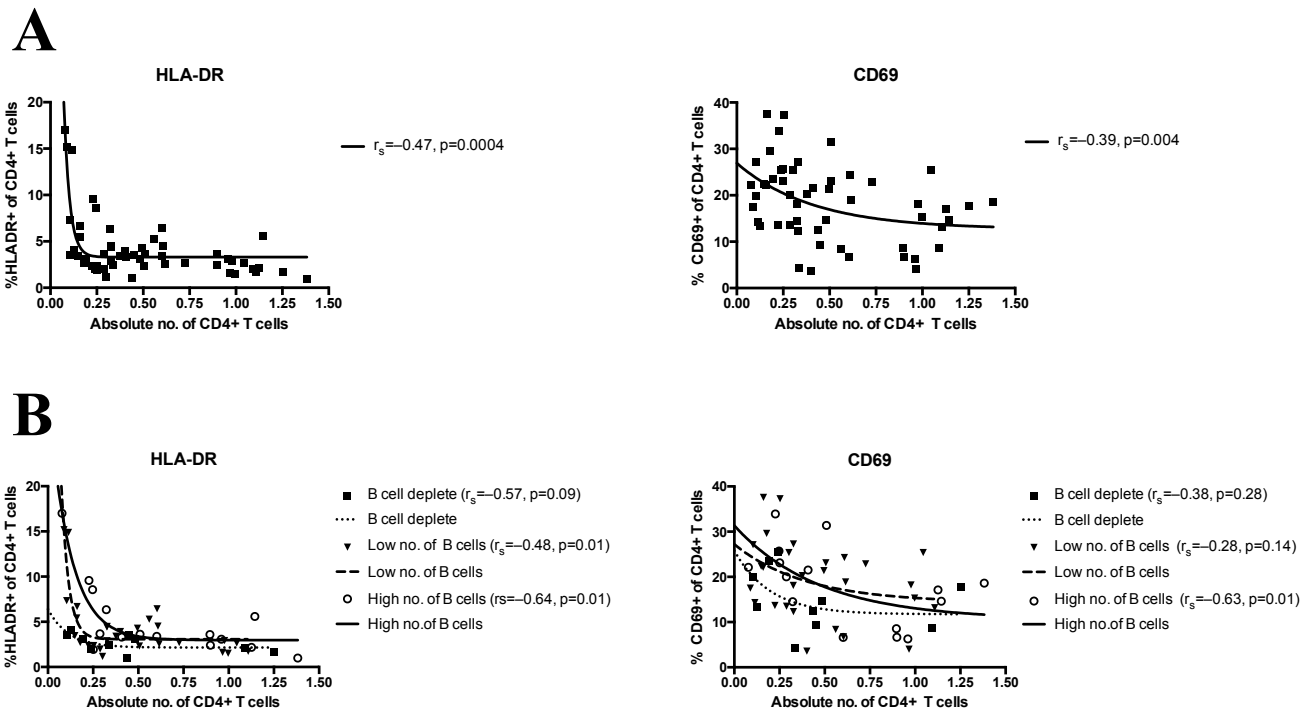


Figure 5.5.4 – Relationship between absolute $CD4^+$ T cell numbers and the percentage of $CD4^+HLA\text{-}DR^+$ T cells and $CD4^+CD69^+$ T cells in the $CD4^+$ T cell pool after BCDT

Data were obtained at least 24 weeks following the last cycle of therapy and shown as scatter plots with lines of best fit, from (**A**) all SLE patients [n = 53], and (**B**) according to whether patients were deplete of B cells [B cell deplete (<0.01 x 10^9/L); n = 10], had low levels of B cells [Low no. of B cells (0.01 to 0.11 x 10^9/L); n = 28] or had high levels of B cells [High no. of B cells (>0.11 x 10^9/L); n = 15]. The Spearman Rank correlation coefficient (r_s) is provided for each association.

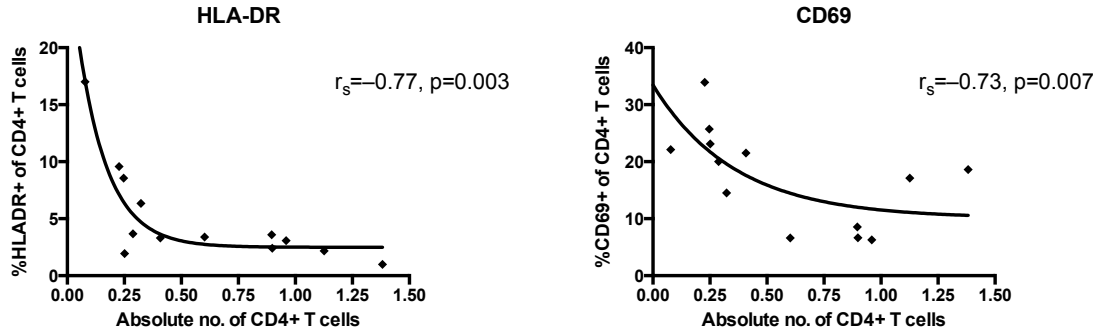


Figure 5.5.5 – Relationship between absolute $CD4^+$ T cell numbers and the percentage of $CD4^+HLA\text{-}DR^+$ T cells and $CD4^+CD69^+$ T cells in the $CD4^+$ T cell pool from SLE patients with high B cell numbers and low anti-dsDNA antibody levels after BCDT

Data were obtained at least 24 weeks following the last cycle of BCDT and shown as scatter plots with lines of best fit, from SLE patients with high B cell numbers (>0.11 x 10^9/L) and low anti-dsDNA antibody levels (<100 IU/L) after BCDT [n = 13]. The Spearman Rank correlation coefficient (r_s) is provided for each association.

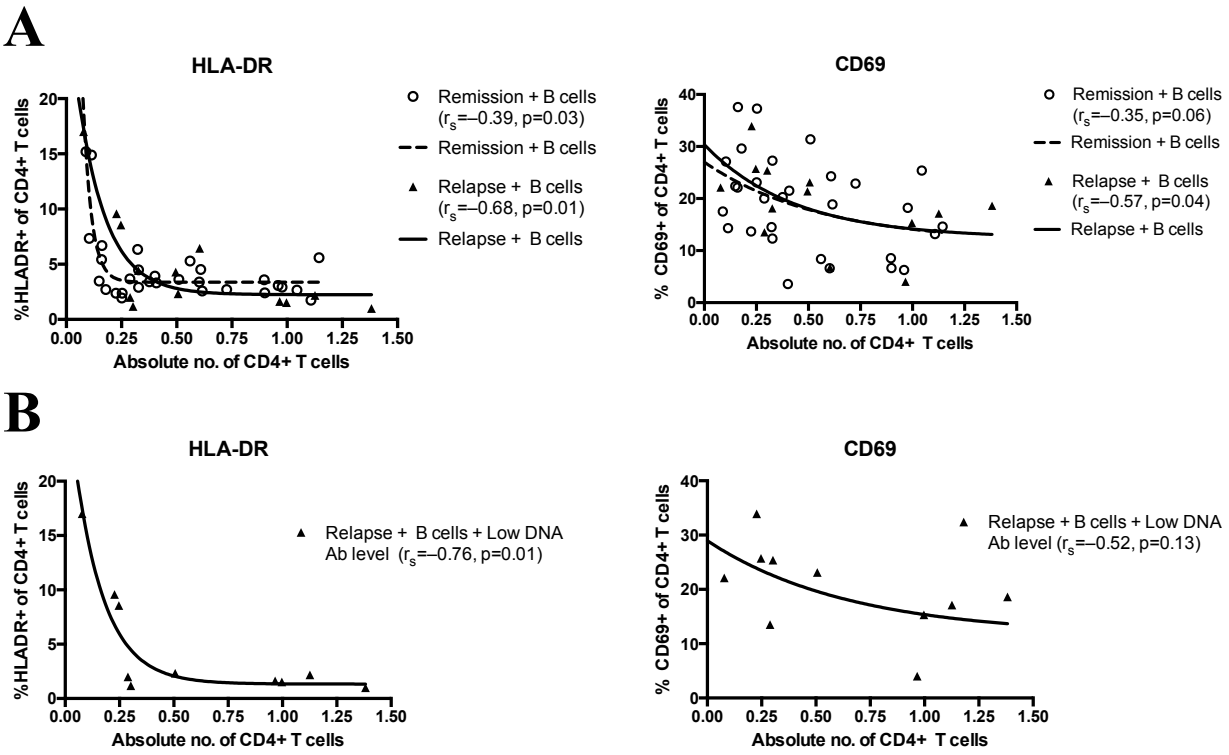


Figure 5.5.6 – Relationship between absolute $CD4^+$ T cell numbers and the percentage of $CD4^+HLA\text{-}DR^+$ T cells and $CD4^+CD69^+$ T cells in the $CD4^+$ T cell pool after B cells have repopulated following BCDT

Data were obtained at least 24 weeks following the last cycle of BCDT and shown as scatter plots with lines of best fit, from SLE patients with B cell numbers >0.01 x 10^9/L from (**A**) SLE patients with active disease [Relapse + B cells; n = 13] or in remission [Remission + B cells; n = 30] and (**B**) in SLE patients patients with B cell numbers >0.01 x 10^9/L with low anti-dsDNA antibody levels (<100 IU/L) and active disease [n = 10]. Data are shown as scatter plots with lines of best fit. The Spearman Rank correlation coefficient (r_s) is provided for each association.

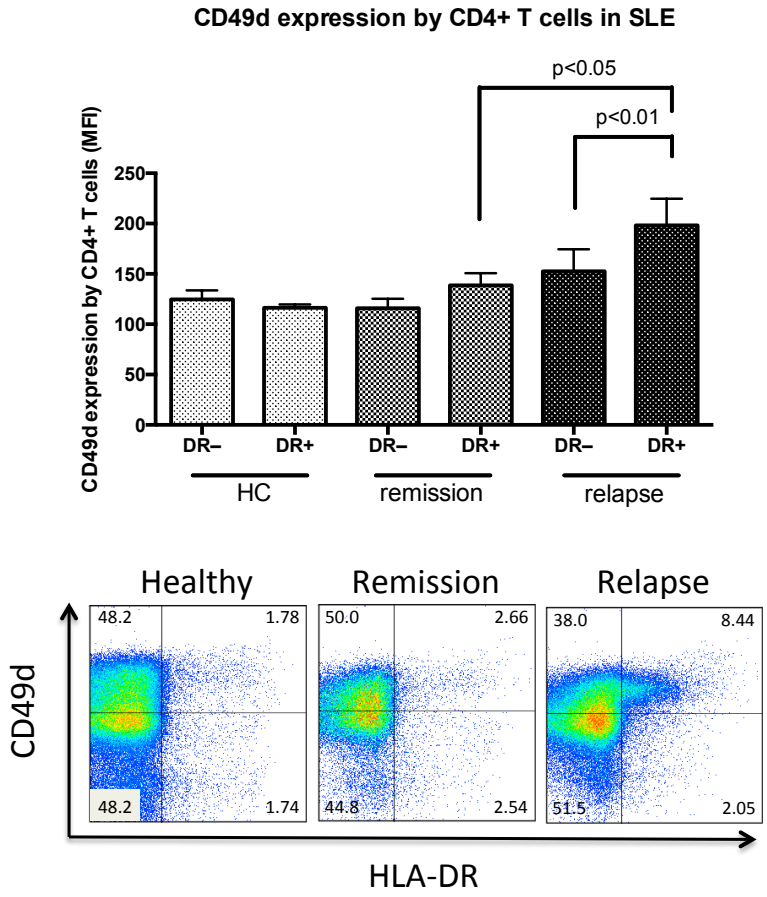


Figure 5.5.7 – CD49d expression by CD4$^+$ T cells in patients with SLE treated with BCDT

Peripheral blood CD4$^+$ T cells were analysed by flow cytometry for the expression of HLA-DR and CD49d. Mean fluorescence intensity (MFI) of CD49d expression by CD4$^+$HLA-DR$^-$ and CD4$^+$HLA-DR$^+$ T cells from age- and sex-matched healthy controls [HC; n = 10], SLE patients in remission post-BCDT [remission; n = 25] and SLE patients at relapse post BCDT [relapse; n = 13] are indicated. Columns indicate the means; bars indicate the S.E.M. Differences between groups were analysed by the Mann-Whitney U test for unpaired data and Wilcoxon matched-pairs signed rank test for paired data. Representative two-parameter dot plots obtained by flow-cytometry are shown.

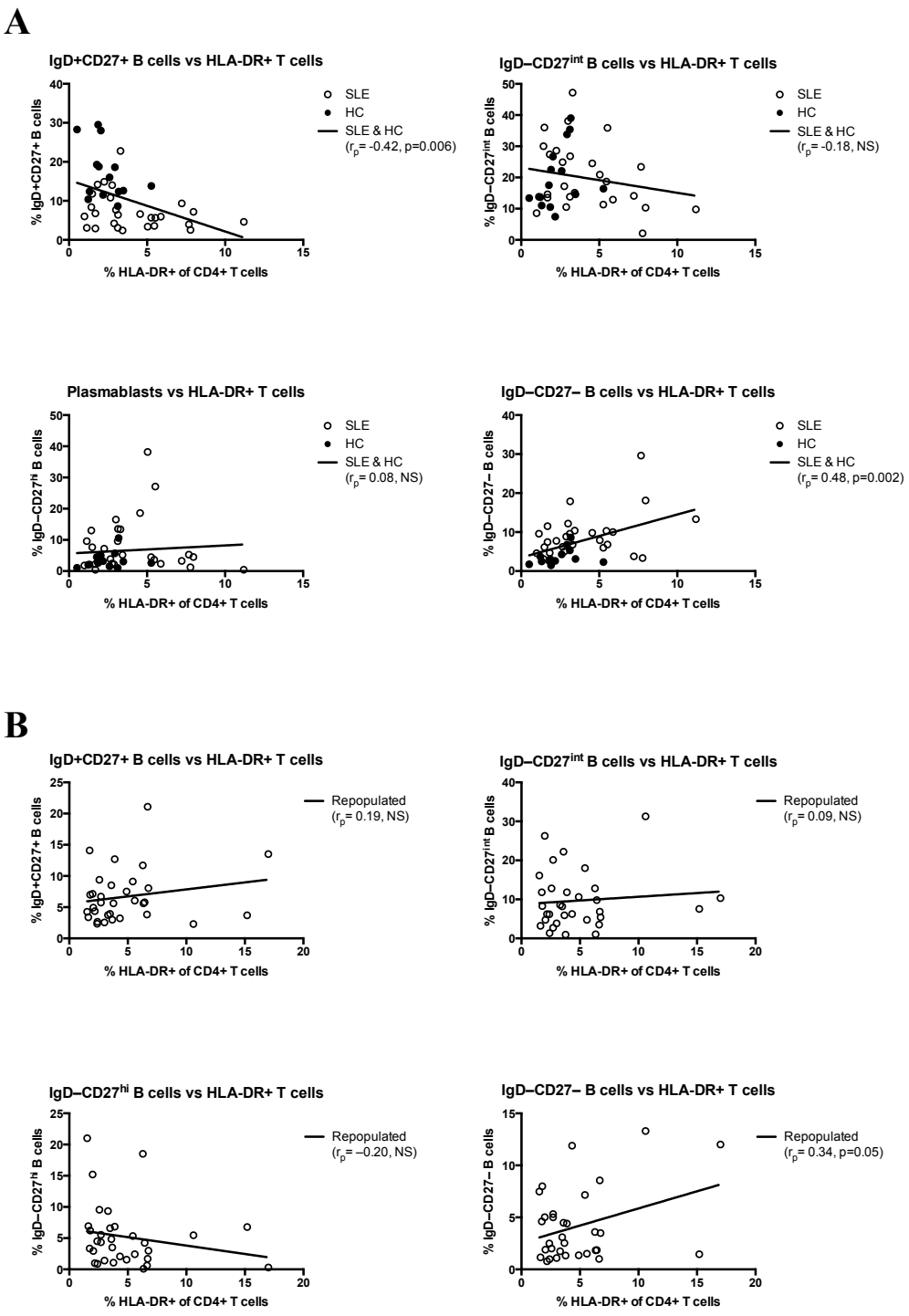


Figure 5.5.8 – Relationship between the percentage of $CD4^+HLA\text{-}DR^+$ T cells in the peripheral $CD4^+$ T cell pool and the percentages of $CD19^+$ B cell memory subsets before and after BCDT

$CD19^+$ B cell memory subsets were identified using the surface markers IgD and CD27; non-switched memory, IgD^+CD27^+, switched-memory, IgD^-CD27^{int}, plasmablasts, IgD^-CD27^{hi} and atypical memory, IgD^-CD27^-. (**A**) Pre-BCDT data from age- and sex-matched healthy controls [HC; n = 14] and patients with SLE who had not been treated with rituximab [SLE; n = 41]. (**B**) Post-BCDT data from SLE patients who had been treated with rituximab at least 24 weeks previously [Repopulated; n = 32]. Data are represented as scatter plots with lines of best fit. The Pearson correlation coefficient (r_p) is provided for each association. NS = $p > 0.05$.

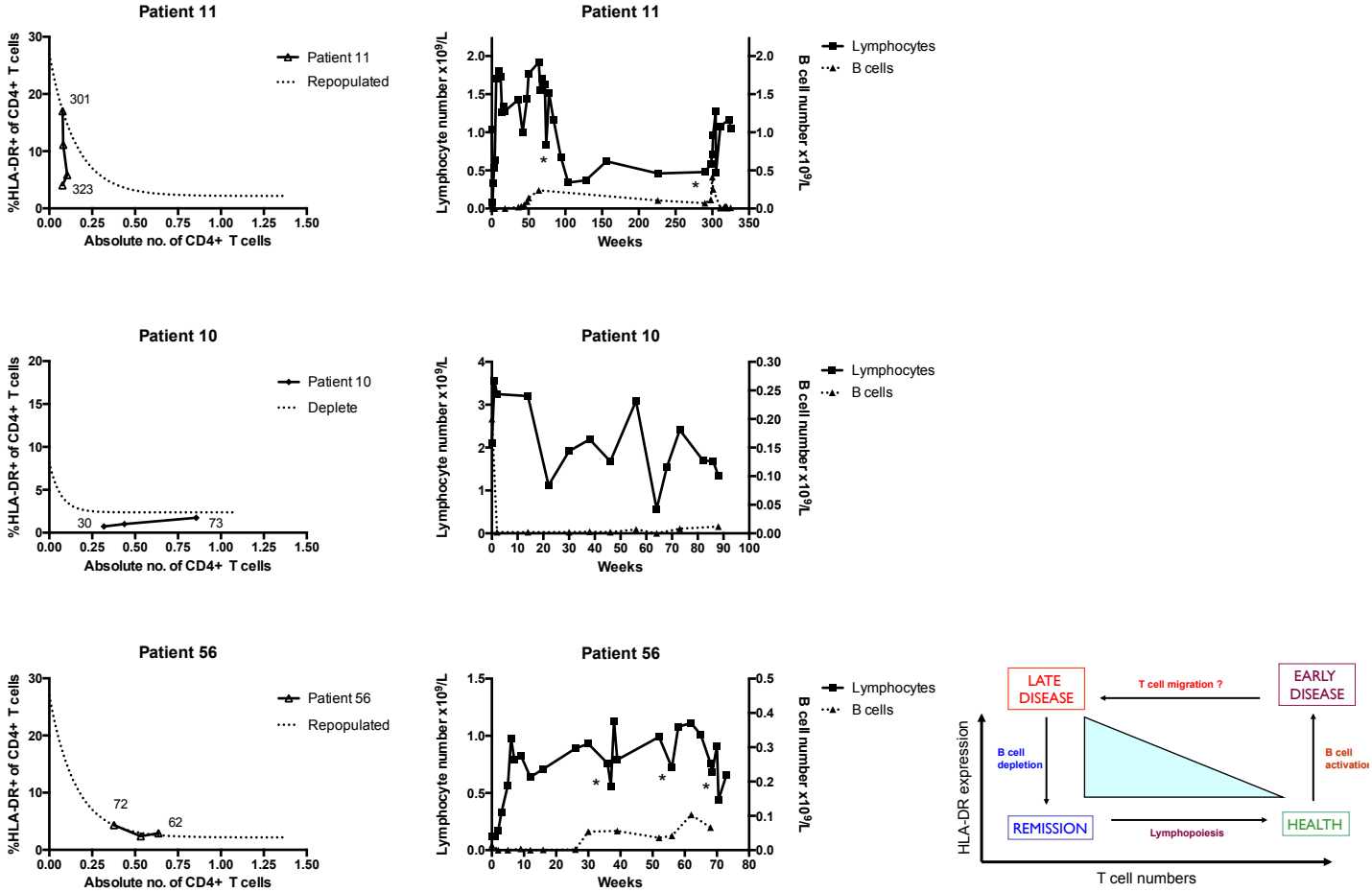


Figure 5.5.9 – Comparison of the changes in HLA-DR expression by $CD4^+$ T cells and changes in $CD4^+$ T cell and total lymphocyte numbers following BCDT

Illustration of the three stages after BCDT – early depletion (first 24 weeks), late depletion (after 24 weeks) and B cell repopulation - showing the relationship between changes in $CD4^+$ T cell numbers with changes in HLA-DR expression by $CD4^+$ T cells when B cells are either deplete (patients 10 and 11) or repopulating (patient 56). HLA-DR expression of $CD4^+$ T cells and $CD4^+$ T cell numbers from different time-points after BCDT are shown with matched regression curves for patients who are deplete of B cells or have repopulated with B cells (left column) are shown with the changes in lymphocyte and B cell numbers (right column) in three patients who have been treated with BCDT. Patient 11 demonstrates changes that occur during the first 24 weeks following her second cycle of BCDT at week 301. Patient 10 demonstrates changes during a period of B cell depletion at least 24 weeks after her last course of BCDT. Patient 56 demonstrates changes following a relapse in disease after B cells have repopulated. The numbers in the figures on the right correspond with time-points (in weeks) in the graphs on the left. An asterisk (*) marks disease flare. A dashed arrow marks retreatment with BCDT. The figures illustrate the dynamic relationship between activated T cells leaving the circulation and non-activated T cells entering the circulation and suggest that B cells are involved in the homeostatic control of circulating T cell numbers (shown in the schematic). $CD49d^{hi}HLA\text{-}DR^+$ T cells, which are induced by IgD^-CD27^- B cells, leave the circulation by migrating through the vascular endothelium whilst naïve $HLA\text{-}DR^-$ T cells enter the circulation after being produced by the bone marrow.

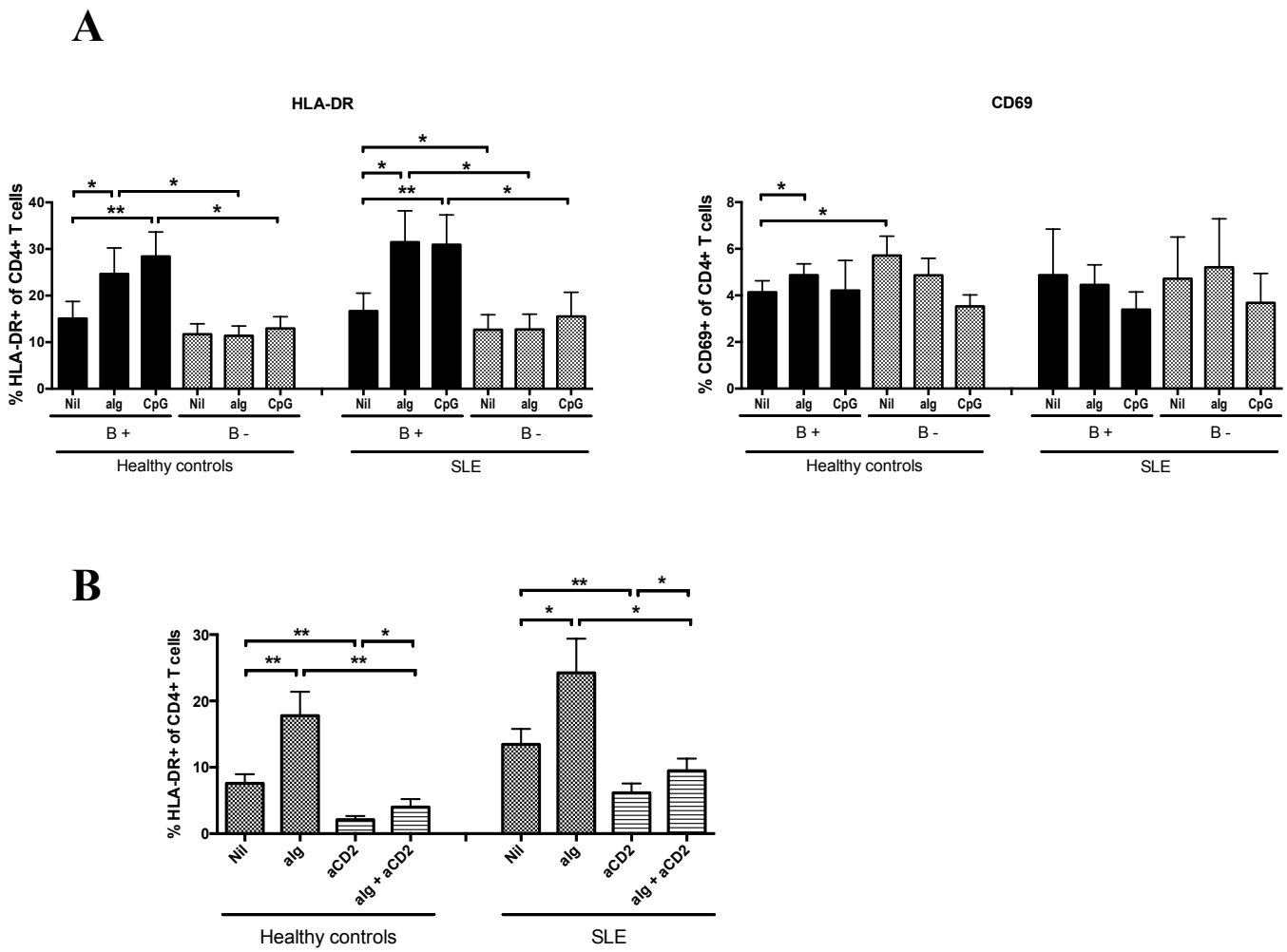


Figure 5.5.10 – Activation of $CD4^+$ T cells by B cells *in vitro*

(**A**) Changes in the expression of HLA-DR and CD69 by $CD4^+$ T cells by depleting B cells from PBMCs of healthy individuals [n = 5] and patients with SLE [n = 5]. B cells were depleted by incubating the PBMCs with antibodies to CD19 attached to MicroBeads and passing the cells through magnetic columns. 2.5 x 10^6 cells were cultured in 200 μl of complete medium (RPMI with 10% foetal calf serum) for 96 hours with either no stimulant (Nil), 2.5 μg/ml $F(ab')_2$ anti-IgM (aIg) or 2 μM CpG 2006 (CpG). PBMCs with B cells are shown in black columns (B+) and without B cells are shown in grey columns (B–). Data are shown as the mean ± SEM. Differences between the groups were analysed by paired t test. * $p < 0.05$, ** $p < 0.01$. (**B**) Changes in the expression of HLA-DR by $CD4^+$ T cells in PBMCs of healthy individuals [n = 6] and patients with SLE [n = 6] following inhibition of LFA-2 (CD2). 2.5 x 10^6 cells were cultured in 200 μl of complete medium (RPMI with 10% foetal calf serum) for 96 hours with either no stimulant (Nil), 2.5 μg/ml $F(ab')_2$ anti-IgM (aIg), 2 μg/ml anti-CD2 (aCD2) or both. PBMCs cultured without anti-CD2 are shown in dark columns and with anti-CD2 in light columns. Data are shown as the mean ± SEM. Differences between the groups were analysed by paired t test. * $p < 0.05$, ** $p < 0.01$.

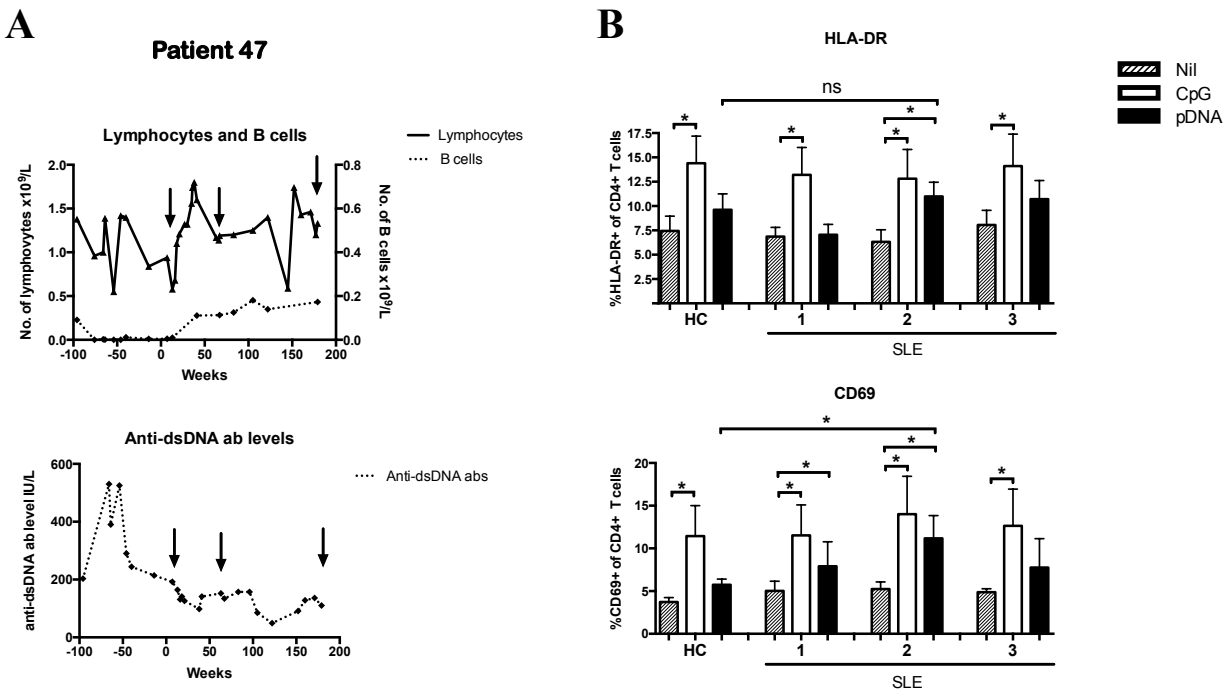


Figure 5.5.11 – Activation of $CD4^+$ T cells by anti-dsDNA antibodies *in vitro*

(**A**) Illustration of the parallel changes in lymphocyte and B cell numbers with the changes in anti-dsDNA antibody levels following BCDT in patient 47. BCDT starts at week 0. Lymphocyte numbers (left Y axis) and B cell numbers (right Y-axis) are both shown using a linear scale. A black arrow is shown when intramuscular methylprednisolone or a reducing course of high dose oral prednisolone was given for a flare of disease. Anti-dsDNA antibody levels are shown using a linear scale. (**B**) Changes in the expression of HLA-DR and CD69 by $CD4^+$ T cells following co-culture of healthy PBMCs with SLE serum and plasmid DNA. PBMCs were obtained from healthy individuals [n = 6]. 2.5 x 10^6 cells were cultured in 200 μl of medium (RPMI with 10% heat-inactivated human serum) for 72 hours with either no stimulant (Nil), 2μM CpG 2006 (CpG) or 10 μg/ml plasmid DNA (pDNA). Serum was obtained from one healthy individual as a control and three SLE patients with high anti-dsDNA antibody levels. Differences between groups were analysed by Wilcoxon matched-pairs signed rank test. NS = $p > 0.05$, * $p < 0.05$.

5.5.3 Discussion

Two studies showed that lymphopaenia (a lymphocyte count of less than 1.5 x 10^9 cells/L) was seen in 75% and 82% of patients with active SLE at the time of presentation (369, 512), but in one of the studies most of the remaining patients also developed lymphopaenia during another episode of active SLE after the initial presentation. The cumulative frequency of lymphopaenia in the course of SLE in this study was 93%. It is the most prevalent laboratory abnormality at presentation, although there is disagreement about whether it predicts (513) or develops during disease exacerbations (370).

A large multicentre study showed that lymphopaenia at presentation is positively associated with anti-dsDNA and Ro antibodies, renal disease, higher disease activity and damage accrual (368). Separate studies of patients with cutaneous disease have shown an association between lymphopaenia and antibodies to Ro, La and snRNPs (372) and with systemic disease (514). The association with systemic disease is consistent with observations seen in the five cases of cutaneous disease that were described in chapter 5.4.

Lymphocytotoxic antibodies were first shown to be present in the serum of SLE patients over 40 years ago (515), but their precise specificity and how they induce cell death has not been established. Some autoantibodies have been shown to bind to surface proteins on lymphocytes, including the surface protein CD4, but these autoantibodies have not been shown to have cytotoxic effects and, therefore, might not cause lymphocyte depletion (376, 516, 517). One study has shown that two out of 25 anti-dsDNA antibodies produced from human-human hybridomas have lymphocytotoxic activity, but as most did not they are unlikely to be the cause of lymphopaenia in the majority of patients (375).

Other mechanisms of lymphocytotoxicity have been suggested, including complement-induced lysis (518), apoptosis (519) and viral destruction of T cells (520). Two studies have suggested that lymphopaenia might be due to lymphocyte sequestration into the tissue, but only one study has provided experimental data to support the hypothesis (384, 520). This is

surprising because there is now a large amount of evidence that lymphocytes, T cells in particular, infiltrate the tissue during active disease. The possibility that T cells can migrate into the tissue, whilst also being lysed by antibodies or dying by other mechanisms seems contradictory. Lymphopaenia in SLE is mostly due to low numbers of $CD3^+$ T cells because they make up approximately 80% of all circulating lymphocytes. There is also evidence of a greater decrease in $CD3^+$ T cells compared to B cells in active disease. It, therefore, seems logical that T cell sequestration into the tissue or SLOs is the cause of lymphopaenia.

There have been several studies that have examined the mechanisms of T cell migration in systemic autoimmune diseases, although most use animal models (521). My observation that HLA-DR expression inversely correlates with $CD4^+$ T cell numbers suggests that it has a functional role in T cell migration. This notion is supported by studies that show a high percentage of HLA-DR expression by T cells in the inflamed tissue, including in the renal interstitium and skin (297, 522). High frequencies of circulating $CD8^+HLA\text{-}DR^+$ T cells has been shown to correlate with NPLE (523) and infiltration of the kidneys by $CD8^+$ T cells (155). Another study showed profound lymphopaenia with high expression of HLA-DR by $CD8^+$ T cells in patients with SLE (297). The observation that HLA-DR is co-expressed with CD49d in patients with active disease is consistent with the observation that $CD4^+$ T cells from patients with scleroderma that migrate through endothelium are more likely to express HLA-DR and also express CD49d, in addition to other adhesion molecules (524).

HLA-DR is a T cell activation marker. It might, therefore, be suggested that T cells are disappearing from the blood due to activation-induced cell death. However, T cells that have increased susceptibility to elimination by activation-induced cell death do not express higher levels of HLA-DR (525). Activation-induced cell death would also fail to account for the increase in lymphocytes, including $HLA\text{-}DR^+$ lymphocytes, which are seen in the inflamed tissue during active disease.

To my knowledge, HLA-DR expression has not previously been shown to induce the migration of T cells but studies show that ligation of HLA-DR molecules on $CD4^+$ T cells increases adhesion with other T cells by upregulating the adhesion pathways between the cells, including the LFA-2/LFA-3 interaction (526, 527). As mentioned in chapter 5.4, T cells are often seen in tissue without other cells present (152, 153) and it was suggested that T cell aggregation is the first stage of ELT formation. The fall in circulating lymphocyte numbers possibly reflects the aggregation of T cells and the formation of ELT. This is supported by the observation in this study of anti-dsDNA antibody levels rising shortly after the lymphocyte numbers fall in patients with low anti-dsDNA antibody levels at remission. It is also supported by observations in this and another study of HLA-DR expression by $CD4^+$ T cells preceding the rise in anti-dsDNA antibody levels (502). It is therefore possible that the B cell induced HLA-DR expression by T cells initiates the formation of ELT.

IgD^-CD27^- B cells are atypical memory B cells that have been shown to be associated with SLE (238, 271), but their function and role in the disease has not been clearly established. The data from this study provides evidence to suggest that IgD^-CD27^- B cells or their precursors might be transferring HLA-DR onto the surface of $CD4^+CD49d^{hi}$ T cells, which increases the ability of T cells to adhere to other lymphocytes and potentially then aggregate in non-lymphoid tissue. This process would be limited by the number of IgD^-CD27^- B cells present, providing a possible explanation for the observation from chapter 5.1 that patients with low anti-dsDNA antibody levels relapse with higher B cell numbers than patients with high anti-dsDNA antibody levels. The results also provide the first evidence of an antibody-independent role for B cells in causing human disease and supports the study of lupus-prone mice that showed T cell infiltration of the renal interstitium that was dependent on a non-antibody secreting B cell (195).

The role of the small collection of perivascular T cells that were seen in the patients with high anti-dsDNA antibody levels in chapter 5.4 is less clear. It is possible that they make the endothelium more permeable to antibodies. This hypothesis is supported by studies of neural

inflammation that showed disruption of the tight junctions between endothelial cells where activated non-neural specific T cells accumulated, allowing the development of autoantibody associated nerve damage (445-447). Endothelial cells can present antigen to T cells, including autoantigen, resulting in their activation (528, 529). What induces the binding of T cells to endothelial cells in SLE is not clear, but several factors that have been associated with active SLE can upregulate the expression of adhesion molecules by endothelial cells, including ultraviolet light (530), pro-inflammatory cytokines (531, 532), anti-dsDNA antibodies (159), and antiphospholipid antibodies (160). Intermittent upregulation of adhesion molecules on endothelial cells by these factors might explain the sudden small fall in circulating lymphocyte numbers during clinical relapse that was seen in the patient with high anti-dsDNA antibodies prior to clinical relapse (patient 47).

A new proposed model of peripheral inflammation is shown (Figure 5.5.12) that suggests that amongst patients with low anti-dsDNA antibody levels at remission tissue inflammation starts with lymphocyte migration, first T cells then B cells and FDCs, which create ELT from where anti-dsDNA antibody producing plasma cells can form resulting in immune complex deposition in the tissue, mediated by T cell induced dysregulation of the endothelial cells. Importantly, this pathway can be separated into 10 phases, some of which can possibly be determined by different biomarkers (Table 5.5.1). The process starts with the abnormal clearance of nuclear antigen (phase 1), which causes the activation of APCs, which then induce the differentiation of $CD4^+$ T cells into either Tem or Trm cells (phase 2). These T cells induce a rise in B cell numbers with high percentages of the IgD^-CD27^- B cell subset (phase 3), followed by increasing numbers of $CD4^+CD49d^{hi}HLA\text{-}DR^+$ T cells which is associated with a fall in circulating T cell numbers (phase 4), then a fall in B cell numbers (phase 5), causing ELT to form in the tissue (phase 6) and culminating in a rise in anti-dsDNA antibody levels (phase 7) and immune complex deposition/ vasculitis leading to a fall in complement levels (phase 8 or 9). Thrombosis might then occur, either as a result of endothelial damage or the release of procoagulant factors during the inflammatory process

(533), although it can also occur in the absence of inflammation, particularly in patients with antiphospholipid antibodies (phase 10). The possible use of HLA-DR expressing T cells as a biomarker is supported by a study showing a positive correlation between $CD4^{+}HLA\text{-}DR^{+}$ T cells and SLE disease activity using the SLAM scale (534).

It could possibly be assumed that the plasma cells that arise from this pathway are short-lived because anti-dsDNA antibody levels fall quite soon after BCDT. However, as this disease pathway only applies to patients who have low anti-dsDNA antibody levels at disease remission or during B cell depletion two important questions arise; where are the LLPCs that produce persistent circulating anti-dsDNA antibody levels being synthesized and what is the role of these persistent anti-dsDNA antibodies in the disease?

The possible association between B cell activation of $CD4^{+}CD49d^{hi}$ T cells and the subsequent formation of ELT is intriguing because blockade of VLA-4, using natalizumab, has been tried successfully in other autoimmune diseases where rituximab has also been shown to be beneficial (535), supporting the possibility of a linked mechanism of action between these two factors. It might also explain the reported associations of progressive multifocal leukoencephalopathy, a cerebral infection due to JC virus, with both of these drugs (536).

B cell depletion has also been linked to reactivation of other viruses, including hepatitis B and C, cytomegalovirus, parvovirus, echovirus and varicella-zoster virus (537, 538), and cryptococcal infections in the blood, brain and bone (539-541), usually when co-administered with high dose chemotherapy. It could be suggested that the viral reactivation is due to the chemotherapy, but $IgD^{-}CD27^{-}$ B cells have been shown to increase during infection with respiratory syncytial virus (277), suggesting that B cells have an important anti-viral role. The data from this study and the model proposed suggest that this effect might not only be due to antibody synthesis, but also $IgD^{-}CD27^{-}$ B cells, which might have an important role in activating T cells to survey tissue that cannot be easily penetrated by circulating antibodies.

Studies suggest that B cells from patients with SLE are activated by antigen via the BCR (507, 508) and/or TLR-9 stimulants (509). This study shows that BCR and TLR-9 stimulants have similar effects on the activation of T cells by B cells. It therefore does not establish which specific stimulant, if any, is important in activating T cells in patients with SLE. Future work would examine the signaling pathways of these B cells because this will provide a better understanding of the factors that stimulate them and be a potential target for treatment, particularly in the patients with high expression of CD49d by $CD4^+$ T cells.

CD69 is a C-type lectin protein that is expressed on the surface of T cells at a very early stage of activation. Its expression on $CD4^+$ T cells, which is high in patients with SLE (542), is increased by cytokines such as IFN-α but only substantially when the TCR is also stimulated (511). Whilst B cells present antigen they do not secrete certain cytokines, in particular IFN-α, which might explain why CD69 expression did not decrease after BCDT – although this result contrasts with two previously published studies (290, 294), the reasons for which are not clear. Its expression could be increased by TLR-9 stimulation, but only when PBMCs were cultured in human serum and not when they were cultured in fetal calf serum. The reason for this difference is unclear but it is possibly due to the presence of human immunoglobulins in the human serum, which can activate APCs via Fc receptors.

The ligand for CD69 is not known but animal studies suggest that its expression on $CD4^+$ T cells has an important role in regulating T cells in (543, 544). More interestingly, it has also been shown to regulate the generation of memory T cells, their homing and survival in the bone marrow and assist the production of high affinity antibodies by controlling the homing of LLPC precursors to the bone marrow (545). A role for CD69 in T cell homing is supported by another study showing that it also controls thymic emigration (546). It is not known if it has similar functions in humans but the data from this study show that, like HLA-DR, it might also inversely affect T cell numbers in the circulation. However, whilst HLA-DR appears to induce the migration of T cells into non-lymphoid tissue to form ELT and SLPCs, the animal

studies suggest that CD69 instead increases the migration of T cells from the blood into the bone marrow where it provides help for LLPCs.

Finally, it should be noted that HLA-DR expression by $CD4^+$ T cells from SLE patients and healthy individuals did not differ when PBMCs were cultured *in vitro* but did differ when measured *ex vivo*. The most likely explanation for the different result is that the SLE patients chosen for the *in vitro* study were in remission, and one of the patients had very low numbers of B cells, which lowered the mean.

In conclusion, this study suggests a sequence of events in patients with low anti-dsDNA antibody levels, starting with B cell expansion of the IgD^-CD27^- memory subset, followed by activation of $CD4^+CD49d^{hi}$ T cells resulting in the disappearance of T cells from the circulation and a rise in anti-dsDNA antibody levels, presumably because ELT are formed in the tissue. This accounts for the formation of SLPCs in a subgroup of patients but does not explain the mechanisms that form LLPCs, which are more likely being generated in SLOs. The next study will examine further T cell differences between patients with anti-dsDNA antibodies produced by SLPCs and those produced by LLPCs.

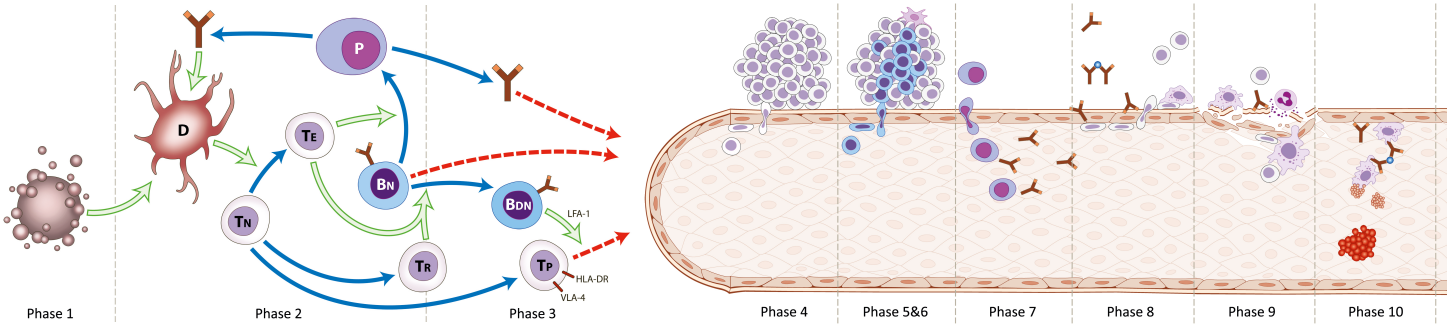


Figure 5.5.12 – Development of anti-dsDNA antibodies in ectopic lymphoid tissue
The inflammatory process of patients with low anti-dsDNA antibody levels during inactive disease can be divided into 10 possible phases, which can be identified by different biomarkers. First there is a rise in circulating B cell numbers, probably $IgD^{-}CD27^{-}$ B cells. This is followed by reduced numbers of circulating $CD4^{+}$ T cells. HLA-DR expression by $CD4^{+}$ T cells is inversely correlated with circulating T cell numbers when B cells have repopulated after BCDT. In active disease after BCDT HLA-DR is expressed with CD49d (VLA-4), which is an adhesion molecule that binds to ligands on endothelial cells. Expression of HLA-DR by $CD4^{+}$ T cells can be induced by TLR-9 or BCR stimulated B cells via LFA-1, which is expressed on T cells. After circulating $CD4^{+}$ T cell numbers fall patients might develop symptoms, such as a rash, or they then develop ELT as B cells, and possibly FDCs, aggregate with T cells. There is then a rise in anti-dsDNA antibody levels, which leads to immune complex deposition or vasculitis. The inflammatory process of patients with high anti-dsDNA antibody levels during inactive disease is unclear.

	Immunological event	Biomarker	Symptoms
Phase 1	Release of nuclear antigen	-	None
Phase 2	Dendritic cell activation T cell differentiation	Cytokines (e.g. IFN-alpha) Memory T cell phenotypes	Non-specific symptoms - Fatigue, myalgia
Phase 3	Expansion of $IgD^{-}CD27^{-}$ B cells Activation of T cells	Rising circulating B cell numbers with high % of $IgD^{-}CD27^{-}$subset High % of $CD4^{+}Cd49d^{h}iHLA\text{-}DR^{+}$ cells	
Phase 4	T cell infiltration of non-lymphoid tissue	T Lymphopaenia	Mild focal symptoms - Malar rash, arthritis, headache
Phase 5	B cell infiltration of non-lymphoid tissue – creating B:T aggregates	B Lymphopaenia?	
Phase 6	Follicular dendritic cells join B:T aggregates in non-lymphoid tissue – creating ectopic lymphoid tissue	-	
Phase 7	Formation of short-lived plasma cells	Rising anti-dsDNA antibody levels	Fevers?, arthralgia?
Phase 8	Immune complex deposition	Fall of complement levels	Diffuse rash, serositis, glomerulonephritis
Phase 9	Endothelial damage		Vasculitis
Phase 10	Thrombosis	Antiphospholipid antibodies	DVT, PE, Stroke, Myocardial infarction

Table 5.5.1 – Summary of the sequence of immunological events and correlations with biomarkers and clinical symptoms in patients with low anti-dsDNA antibody levels during inactive disease

5.6 Chemokine receptor expression by $CD4^+$ T cells differs according to how anti-dsDNA antibody levels change after BCDT in SLE

5.6.1 Introduction

In chapter 5.1 it was suggested that there are three patterns of disease defined by the levels of anti-dsDNA antibodies and how they change following BCDT; active disease with low anti-dsDNA antibodies but with high B cell numbers with a high percentage of IgD^-CD27^- B cells, active disease with high anti-dsDNA antibody levels that are high during the period of active disease and fall during periods of remission and active disease with persistently elevated anti-dsDNA antibodies that do not correlate with disease activity. Examples of these three patterns of clinical disease were provided by the case studies in chapter 5.3. In chapter 5.4 it was shown that there are histological differences in the inflamed tissue with some possible links to the anti-dsDNA antibody level, with patients with low levels having more T cell infiltration. However, anti-dsDNA antibodies appeared later in the patient that also had B cells in the infiltrate suggesting that T cell infiltration is the first stage in the formation of ELT. In chapter 5.5, it was shown that B cell repopulation is associated with a fall in circulating T cell numbers, which correlates with the expression of HLA-DR by $CD4^+CD49d^{hi}$ T cells. HLA-DR expression by $CD4^+$ T cells correlated with the percentage of IgD^-CD27^- B cells, suggesting that these cells are inducing the migration of $CD4^+$ T cells.

In most patients there was a rise in anti-dsDNA antibody levels shortly after T cell numbers fell, supporting the hypothesis that these T cells are migrating to form ELT. It would appear that the plasma cells that produce these anti-dsDNA antibodies are short-lived, as the anti-dsDNA antibody levels fall after BCDT. By contrast anti-dsDNA antibodies that persist after BCDT are most likely secreted by long-lived plasma cells (LLPC) (547). Studies of humans and mice with autoimmune disease have shown that these LLPCs mostly reside in SLOs (548, 549), but they have also been found in the inflamed kidneys of mice with lupus-like disease (550). Repeat biopsies of kidneys from SLE patients treated with rituximab have shown that plasma cells remain present in the tissue even when B cells are absent (441). However, it is

not known if the LLPCs that are seen in the peripheral tissue are arising from ELT or migrating from SLOs.

It was hypothesized that if anti-dsDNA antibodies that disappear after BCDT are initially arising from ELT then the time at which they reappear would depend on the rate of T cell migration to the non-lymphoid tissue and the number of circulating $IgD^{-}CD27^{-}$ B cells to activate them, which was shown to correlate with the proportion of circulating revertant memory T cells (Trm) and to a lesser extent effector memory T cells (Tem) in chapter 5.2.

The proposed model suggests that patients with levels that rise and fall depending on the severity of the disease would have T cells that migrate to non-lymphoid tissue, whilst those patients that have high anti-dsDNA antibody levels that never return to normal, even during remission, would have T cells that migrate to SLOs.

T cells from patients with SLE have been shown to express a number of different chemokine receptors, some which guide migration to the non-lymphoid tissue and others that guide migration to SLOs. I therefore decided to compare the expression of chemokine receptors and adhesion molecules of $CD4^{+}$ T cells in patients depending on the levels of their anti-dsDNA antibody levels during active disease and remission and their memory T cell subsets.

It was also hypothesized that there might be differences between patients depending on whether their B cells were likely to repopulate early with high percentages of $IgD^{-}CD27^{-}$ B cells or late after BCDT, which can be predicted by the percentage of revertant (Trm) or effector (Tem) memory T cell subsets as was shown in chapter 5.2.

5.6.2 Results

Patients were divided into three columns according to the levels of their anti-dsDNA antibody levels during active and inactive disease; if they rise with disease activity and then fall below 50 IU/L during remission (1), if they remain <50 IU/L regardless of disease severity (2), or remain elevated even during remission (3). Each column was divided into three rows according to their percentage of memory T cell as previously described in chapter 2; patients with revertant memory T cells >0.8% (A), with effector memory T cells >3% and Trm <0.8% (B) or with neither high Trm or Tem cells (C), thus creating 9 groups in total.

Chemokine receptors

The expression of chemokine receptors and adhesion molecules of $CD4^+$ T cells by $CD4^+$ T cells were compared between the 9 groups (Table 5.6.1). As the precise pathology of group 2 was unclear it was decided to compare them between columns 1 and 3, who were hypothesized to have disease due to plasma cells from ELT and SLOs respectively.

The chemokine receptors and adhesion molecules that were examined were CXCR3, CCR4, CCR5, CCR7 with CD62L, CD49d by $CD4^+$ T because they have all been shown to be increased in different subgroups of patients with SLE. Studies show that terminally differentiated effector memory T cells have higher percentages of cells that express receptors that are specific for chemoattractants to non-lymphoid tissue, such as CXCR3, CCR4 and CCR5, and have lower percentages of cells that express receptors for chemoattractants to SLOs, such as CCR7 and CD62L. However, it was hypothesized that patients with persistently elevated anti-dsDNA antibody levels due to LLPCs from SLOs would have a higher percentage of $CD4^+$ T cells expressing CCR7 and CD62L, whilst patients with levels of anti-dsDNA antibodies that return to normal after BCDT, and are therefore being produced by SLPCs from ELT, would have a higher percentage of $CD4^+$ T cells expressing CXCR3, CCR4, CCR5 and CD49d.

Comparisons of columns within each row showed that the percentage of $CCR7^+CD62L^+$ $CD4^+$ T cells was significantly lower in column 1 compared to column 3 in rows A ($p = 0.0001$) and B ($p = 0.04$). The expression of CD49d by $CD4^+$ T cells was significantly higher in group 1 compared to group 3 in the rows A ($p = 0.0007$) and B ($p = 0.04$). The percentage of $CXCR3^+$ and $CCR5^+$ $CD4^+$ T cells was significantly higher in column 1 compared to group 3 but only in row A ($p = 0.007$ for CXCR3 and $p < 0.0001$ for CCR5). The percentage of $CCR4^+$ $CD4^+$ T cells did not differ between the columns.

Comparisons of rows within each column showed that the percentage of $CCR7^+CD62L^+$ $CD4^+$ T cells was significantly lower in row A compared to C row in columns 1 ($p < 0.0001$) and 2 ($p = 0.001$) but not in column 3. The expression of CD49d by $CD4^+$ T cells was significantly higher in the row A compared to rows B ($p < 0.0001$) and C ($p = 0.0005$) but only in the column 1. The percentage of $CXCR3^+$ $CD4^+$ T cells was significantly higher in row A compared to row C in the columns 1 ($p = 0.002$) and 2 ($p = 0.04$) but not in column 3. The percentage of $CCR5^+$ $CD4^+$ T cells was significantly higher in row A compared to row C in column 1 only ($p < 0.0001$). The percentage of $CCR4^+$ $CD4^+$ T cells did not differ between the rows.

Comparisons of groups regardless of rows and columns showed that the mean percentage of $CXCR3^+$ and $CCR5^+$ $CD4^+$ T cells and the mean level of expression of CD49d by $CD4^+$ T cells was highest in the group A1, particularly when compared with group C1 ($p = 0.02$ for CXCR3, $p = 0.0001$ for CCR5 and $p = 0.005$ for CD49d), group C3 ($p = 0.007$ for CXCR3, $p < 0.0001$ for CCR5 and $p = 0.005$ for CD49d) and group A3 ($p = 0.06$ for CXCR3, $p = 0.0002$ for CCR5 and $p = 0.007$ for CD49d). However, there was no significant difference in the expression of CXCR3, CCR5 and CD49d by $CD4^+$ T cells of patients in the C3 group compared to patients in the A3 group. The mean percentage of $CD4^+$ T cells expressing CCR4 did not differ between any of the group. The mean percentage of $CCR7^+CD62L^+$ $CD4^+$ T cells was lowest in group A1 when compared with groups C1 ($p = 0.001$), C3 ($p < 0.0001$) and A3 ($p < 0.0001$). Similar to the results that were seen with the other chemokine receptors

there was no significant difference in the mean percentage of $CCR7^+CD62L^+$ $CD4^+$ T cells in group A3 compared to group C3.

One-way ANOVA with post test for linear trend showed that there was a significant negative linear trend in the mean percentage of $CXCR3^+$ and $CCR5^+$ $CD4^+$ T cells from the A1 to the A3 ($p = 0.03$ for CXCR3, $p = 0.01$ for CCR5) and the C3 groups ($p = 0.002$ for CXCR3, $p = 0.001$ for CCR5) and a positive linear trend in the mean percentage of $CCR7^+CD62L^+$ $CD4^+$ T cells from the A1 to the A3 ($p = 0.0006$) and the C3 groups ($p < 0.0001$) supporting the hypothesis that column 2 is an intermediate group with patients that share pathology with groups in either columns 1 and 3.

Although CCR4 expression did not differ between the groups, the percentage of $CD4^+$ T cells that express CCR4 was higher in all groups compared to healthy controls ($p < 0.05$, Mann-Whitney U test) except in the A1, A3 and C1 groups. Also, whilst the percentage of $CD4^+$ cells expressing CCR5 did not differ between the groups A3 and C3, there was significant difference in the percentage of $CD4^+CCR5^+$ T cells in the A3 group compared to healthy controls ($p = 0.015$, Mann Whitney U test) but there was not a significant difference between group C3 and healthy controls ($p = 0.26$, Mann Whitney U test).

There is therefore statistically significant difference in the expression of chemokine receptors between the groups, in particular between rows A and B of columns 1 and 3. The $CD4^+$ T cells of patients in column 1 are more likely to express chemokine receptors for non-lymphoid tissue and high levels of CD49d, whilst the $CD4^+$ T cells of patients in column 3 are more likely to express chemokine receptors for SLOs. Statistically significant difference was consistently greatest between groups A1 and the C3 suggesting that they had diametrically opposite T cell migrating patterns, which might explain the differences in the changes in anti-dsDNA antibody levels following BCDT between the two groups. Figure 5.6.1 illustrates the differences in the expression of the chemokine receptors CCR5, CCR7, CD62L, CXCR3 and the adhesion molecule CD49d by $CD4^+$ T cells from patients in the groups A1, A3 and C3.

Disease manifestations

The incidence of clinical manifestations was compared between the groups using Fisher's exact or Chi-squared tests (Table 5.6.2). Glomerulonephritis (WHO Class III to V) occurred most frequently in the columns 1 and 3, which had high anti-dsDNA antibodies ($p = 0.002$ Chi-square test), consistent with other studies. Vasculitis occurred most frequently in column 1 ($p = 0.03$, Chi-square test for trend). Neuropsychiatric disease occurred most frequently in row A ($p = 0.02$, Chi-square test) and was also associated with higher percentages of CCR5 expressing $CD4^+$ T cells when the one case of CNS vasculitis was excluded ($p = 0.03$, Mann Whitney U test) (data not shown). Lymphadenopathy occurred most frequently in column 3 ($p = 0.003$, Chi-square test) and there was significant positive trend as T cells became more differentiated ($p = 0.02$, Chi-square test for trend). However, it is possible that lymphadenopathy was under-recorded.

There were no statistically significant differences in the incidence of cytopaenia, mouth ulcers, serositis, alopecia and rashes between the groups.

Laboratory parameters

Complement levels and leukocyte numbers have been shown to be abnormal in some but not all patients with active disease. I therefore investigated whether there are significant differences in C3 levels, and numbers of lymphocyte and monocytes prior to BCDT between the groups (Table 5.6.3).

Multiple comparisons tests showed that there was a significant difference in the mean C3 level at baseline between column 3 and column 2 ($p = 0.02$). The results are consistent with studies that have shown an association between hypocomplementaemia and high anti-dsDNA antibodies. However, there were no other significant differences between the groups. In particular, patients in column 1, who had high anti-dsDNA antibody levels during active disease that fell after BCDT, had mean C3 levels that were not significantly different to patients in column 2, who had persistently low anti-dsDNA antibody levels.

Comparisons of the mean number of lymphocytes at baseline within each column showed that in column 1 row A had the highest number of lymphocytes compared to rows B ($p = 0.0002$) and C ($p = 0.01$). Similarly comparisons within each row showed that in row A the mean lymphocyte number was highest in column 1 compared to columns 2 ($p = 0.005$) and 3 ($p = 0.009$). Comparisons of groups regardless of rows and columns showed that the mean lymphocyte number was again highest in group A1 compared to groups B1 ($p = 0.002$), A2 ($p < 0.05$), B2 ($p < 0.05$) and C3 ($p = 0.02$).

Comparisons of the mean number of monocytes at baseline did not reveal any statistically significant differences between the groups.

Age at diagnosis

Comparisons of the mean age of diagnosis between the groups regardless of columns and rows showed that patients in group A2 were diagnosed at an older age compared to patients in the B1 ($p = 0.01$) and B3 ($p < 0.05$) groups (Table 5.6.4). Comparisons of rows within each column showed that patients in the A2 group had a significantly higher mean age of diagnosis compared to patients in the C2 group ($p = 0.04$). Comparisons of columns within each row did not reveal any significant differences between the groups.

Response to BCDT

It has already been shown that the median time to relapse following BCDT was lower in patients with more differentiated T cells but there was surprisingly no significant difference in patients with high anti-dsDNA antibody levels compared to patients with low anti-dsDNA antibody levels at the time of treatment. In this study the time to relapse was compared between the 9 groups. A significant difference in survival curves was seen between the groups ($p = 0.02$, log-rank (Mantel-Cox) test) (Table 5.6.5). Group A3 had the lowest median time to relapse of 8 weeks, whilst groups C1 and C3 had the highest median times to relapse of 287 and 295.5 weeks respectively. Thus it shows that the time to clinical relapse is associated with several $CD4^+$ T cell factors, including the memory T cell phenotypes, which

correlate with the rate of B cell repopulation, and their expression of chemokine receptors and adhesion molecules, which are associated with different patterns of changes in the anti-dsDNA antibody levels.

		Anti-dsDNA level IU/L		
		Min: 0 to 50 Max: > 50 **1**	Min: 0 to 50 Max: 0 to 50 **2**	Min: >100 Max: >200 **3**
CXCR3$^+$ %	High Trm **A**	**59.9** (-61.5-181.2; 2)	39.9 (20.4-59.4; 4)	31.1 (14.4-47.8; 4)
	High Tem **B**	31.6* (25.9-37.3; 14)	33.4 (26.8-40.0; 8)	27.7** (21.3-34.1; 15)
	Low Tem **C**	13.7* (—;1)	23.5** (14.9-32.1; 7)	22.3** (-10.4-55.1; 3)
CCR7$^+$ CD62L$^+$ %	High Trm **A**	**14.1** (-104-132; 2)	40.0 (28.2-49.8; 4)	58.0** (45.0-70.9; 4)
	High Tem **B**	55.0*** (46.1-63.9; 14)	53.7** (46.4-60.9; 8)	65.3**** (60.0-70.7; 15)
	Low Tem **C**	82.7**** (-8.8-174; 2)	65.0**** (56.4-73.6; 7)	71.8*** (42.5-101; 3)
CD49d MFI	High Trm **A**	**299.0** (197-400; 2)	102.2*** (54.4-150; 4)	147.4** (25.5-269; 4)
	High Tem **B**	145.0** (111-178; 13)	99.2**** (69.9-128; 5)	98.9**** (78.3-119; 10)
	Low Tem **C**	79.8** (—;1)	106.6**** (81.4-132; 7)	118.1** (-172-409; 2)

Table 5.6.1 – Expression of chemokine receptors and adhesion molecules by CD4+ T cells in SLE patients treated with BCDT

Expression of chemokine receptors and adhesion molecules by $CD4^+$ T are shown in groups of patients that are divided according to their memory T cell phenotypes (rows; High Trm (A), High Tem with low Trm (B) or Low Tem (C)) and how anti-dsDNA antibody levels change after BCDT (columns; High at baseline (>50 IU/L) and fall after BCDT (1), Low (2) or Persistently elevated after BCDT (3)). Data shows the mean with 95% confidence interval and numbers in the group in parentheses. Differences between the groups were analysed by two-way ANOVA with Tukey's multiple comparison post-test. Statistical significance is shown for comparisons with group A1 (shown in bold). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

		Anti-dsDNA level IU/L		
		Min: 0 to 50 Max: > 50 **1**	Min: 0 to 50 Max: 0 to 50 **2**	Min: >100 Max: >200 **3**
CCR4$^+$ %	High Trm **A**	19.6 (-44.6-83.7; 2)	37.1 (22.8-51.4; 4)	29.1 (4.9-53.3; 4)
	High Tem **B**	31.7 (22.3-41.1; 14)	**37.9** (30.4-45.4; 8)	27.2 (21.6-32.8; 15)
	Low Tem **C**	19.4 (—;1)	26.1 (21.1-31.1; 7)	28.7 (20.4-36.9; 3)
CCR5$^+$ %	High Trm **A**	**55.9** (-183-295; 2)	19.1**** (8.0-30.3; 4)	20.9*** (11.2-30.3; 4)
	High Tem **B**	16.6**** (11.3-21.9; 14)	16.3**** (11.9-20.7; 8)	11.3**** (8.1-14.6; 15)
	Low Tem **C**	5.8*** (—;1)	9.8**** (6.0-13.6; 7)	13.7**** (-12.3-39.6; 3)

Table 5.6.1 continued

Expression of chemokine receptors and adhesion molecules by CD4$^+$ T are shown in groups of patients that are divided according to their memory T cell phenotypes (rows; High Trm (A), High Tem with low Trm (B) or Low Tem (C)) and how anti-dsDNA antibody levels change after BCDT (columns; High at baseline (>50 IU/L) and fall after BCDT (1), Low (2) or Persistently elevated after BCDT (3)). Data shows the mean with 95% confidence interval and numbers in the group in parentheses. Differences between the groups were analysed by two-way ANOVA with Tukey's multiple comparison post-test. Statistical significance is shown for comparisons with group A1 (shown in bold). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

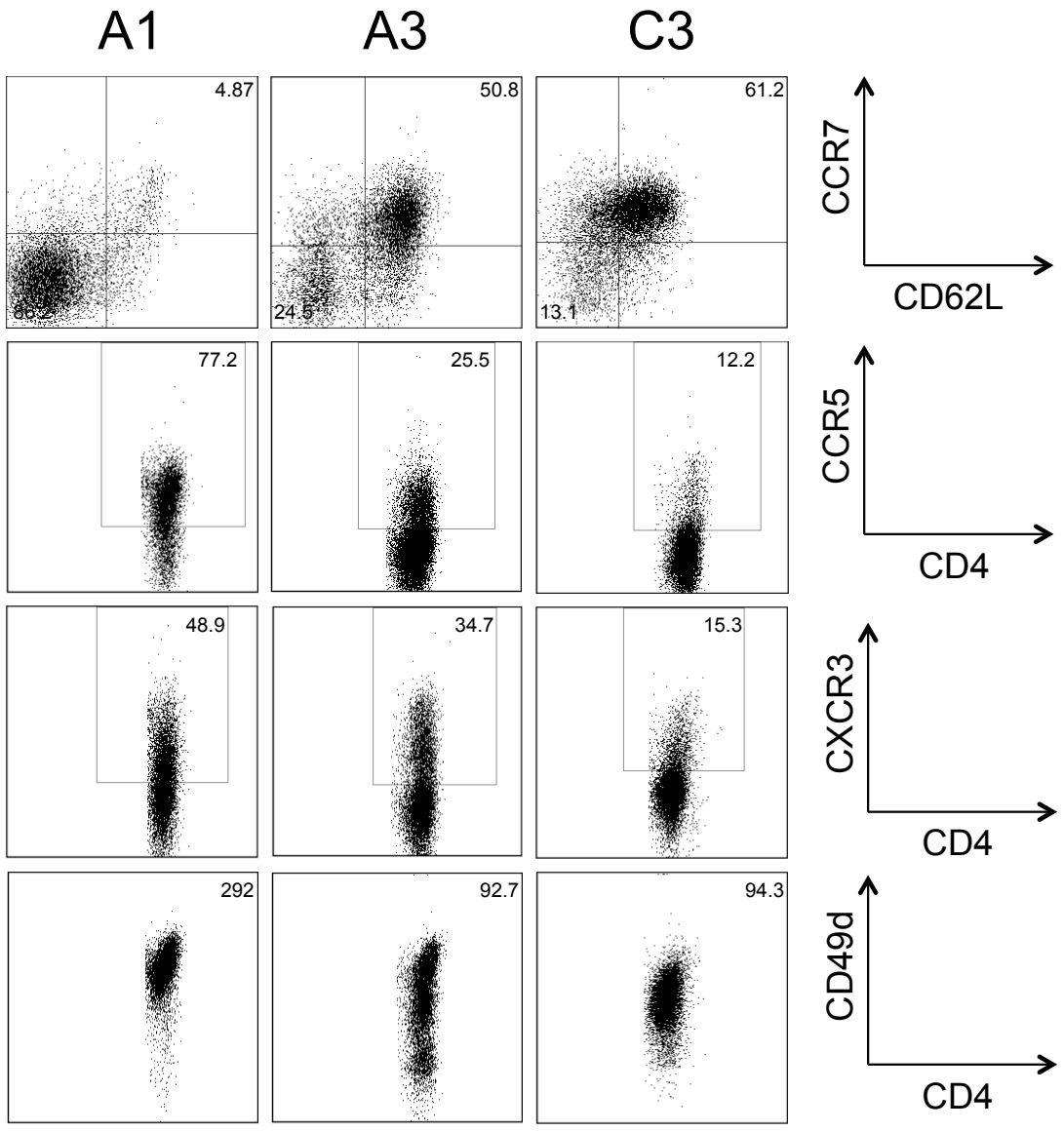


Figure 5.6.1 – Expression of chemokine receptors and adhesion molecules by $CD4^+$ T cells in SLE patients treated with BCDT

Representative flow cytometry dot plots showing the expression of CCR7, CD62L, CCR5, CXCR3 and CD49d by $CD4^+$ T cells from patients in groups A1, A3 and C3. Values represent the percentage of $CD4^+$ T cells expressing CCR7, CD62L, CCR5 and CXCR3 and the mean fluorescence intensity of CD49d expression.

		Anti-dsDNA level IU/L			
		Min: 0 to 50 Max: > 50 **1**	Min: 0 to 50 Max: 0 to 50 **2**	Min: >100 Max: >200 **3**	
Renal (Class III to V)	High Trm **A**	**33** (1/3)	0 (0/5)	**50** (2/4)	p=0.002
	High Tem **B**	**81** (13/16)	25 (2/8)	**47** (7/15)	Chi^2-test
	Low Tem **C**	**0** (0/2)	14 (1/7)	**100** (4/4)	
Vasculitis	High Trm **A**	**0** (0/3)	0 (0/5)	0 (0/4)	p=0.03
	High Tem **B**	**38** (6/16)	13 (1/8)	7 (1/15)	Chi^2-test for tre
	Low Tem **C**	**50** (1/2)	14 (1/7)	25 (1/4)	
NPLE	High Trm **A**	**33** (1/3)	**40** (2/5)	**25** (1/4)	p=0.02
	High Tem **B**	7 (1/16)	25 (2/8)	0 (0/15)	Chi^2-test
	Low Tem **C**	0 (0/2)	0 (0/7)	0 (0/4)	
Lymphadeno pathy	High Trm **A**	0 (0/3)	0 (0/5)	**75** (3/4)	p=0.003
	High Tem **B**	0 (0/16)	0 (0/8)	**20** (3/15)	Chi^2-test
	Low Tem **C**	0 (0/2)	0 (0/7)	**0** (0/4)	

Table 5.6.2 – Clinical symptomatology in SLE patients treated with BCDT
Percentage (numbers) of patients with SLE symptoms are shown in groups that are divided according to their memory T cell phenotypes (rows; Low Tem (N), High Tem with low Trm (E) or High Trm (R)) and how anti-dsDNA antibody levels change after BCDT (columns; High at baseline (>50 IU/L) and fall after BCDT (1), Low (2) or Persistently elevated (3)). Differences between the groups were analysed by Chi-square test or Chi-square test for trend. Rows or columns with significantly higher incidence of symptoms are shown in bold.

		Anti-dsDNA level IU/L		
		Min: 0 to 50 Max: > 50 **1**	Min: 0 to 50 Max: 0 to 50 **2**	Min: >100 Max: >200 **3**
Serositis	High Trm **A**	33 (1/3)	20 (1/5)	25 (1/4)
	High Tem **B**	31 (5/16)	13 (1/8)	33 (5/15)
	Low Tem **C**	100 (2/2)	29 (2/7)	25 (1/4)
Cytopaenia	High Trm **A**	0 (0/3)	20 (1/5)	0 (0/4)
	High Tem **B**	25 (4/16)	0 (0/8)	7 (1/15)
	Low Tem **C**	0 (0/2)	14 (1/7)	0 (0/4)
Mouth Ulcers	High Trm **A**	0 (0/3)	20 (1/5)	25 (1/4)
	High Tem **B**	13 (2/16)	13 (1/8)	13 (2/15)
	Low Tem **C**	0 (0/2)	0 (0/7)	0 (0/4)
Alopecia	High Trm **A**	0 (0/3)	20 (1/5)	25 (1/4)
	High Tem **B**	31 (5/16)	25 (2/8)	13 (2/15)
	Low Tem **C**	0 (0/2)	13 (1/7)	0 (0/4)
Skin	High Trm **A**	33 (1/3)	60 (3/5)	50 (2/4)
	High Tem **B**	56 (9/16)	88 (7/8)	53 (8/15)
	Low Tem **C**	50 (1/2)	43 (3/7)	0 (0/4)

Table 5.6.2 continued

Percentage (numbers) of patients with SLE symptoms are shown in groups that are divided according to their memory T cell phenotypes (rows; Low Tem (N), High Tem with low Trm (E) or High Trm (R)) and how anti-dsDNA antibody levels change after BCDT (columns; High at baseline (>50 IU/L) and fall after BCDT (1), Low (2) or Persistently elevated (3)). Differences between the groups were analysed by Chi-square test or Chi-square test for trend. Rows or columns with significantly higher incidence of symptoms are shown in bold.

		Anti-dsDNA level IU/L		
		Min: 0 to 50 Max: > 50 **1**	Min: 0 to 50 Max: 0 to 50 **2**	Min: >100 Max: >200 **3**
C3	High Trm **A**	**1.10** (-0.50-2.71; 3)	1.13 (0.54-1.73; 5)	0.58 (-0.32-1.48; 3)
	High Tem **B**	0.73 (0.58-0.87; 16)	0.88 (0.49-1.26; 7)	0.72 (0.57-0.87; 15)
	Low Tem **C**	0.85 (-4.68-6.37; 2)	0.93 (0.70-1.15; 7)	0.58 (0.29-0.87; 4)
Lymph	High Trm **A**	**2.43** (-1.81-6.66; 3)	0.98* (0.49-1.47; 5)	0.91 (0.82-1.01; 3)
	High Tem **B**	0.76** (0.50-1.03; 16)	1.07* (0.61-1.53; 7)	1.27 (0.87-1.66; 13)
	Low Tem **C**	0.81 (-0.97-2.59; 2)	1.70 (1.39-2.02, 7)	0.74* (-0.17-1.66; 4)
Mono	High Trm **A**	**0.62** (0.29-0.94; 3)	0.40 (0.14-0.66; 5)	0.33 (-0.14-0.81; 3)
	High Tem **B**	0.40 (0.28-0.52; 16)	0.42 (0.26-0.57; 7)	0.47 (0.33-0.62; 11)
	Low Tem **C**	0.23 (-0.09-0.54; 2)	0.54 (0.32-0.75; 7)	0.29 (-0.18-0.76; 4)

Table 5.6.3 – Baseline complement levels and circulating numbers of lymphocytes and monocytes of SLE patients treated with BCDT

Baseline complement levels and numbers of lymphocytes and monocytes are shown in groups of patients that are divided according to their memory T cell phenotypes (rows; Low Tem (N), High Tem with low Trm (E) or High Trm (R)) and how anti-dsDNA antibody levels change after BCDT (columns; High at baseline (>50 IU/L) and fall after BCDT (1), Low (2) or Persistently elevated (3)). Data shows the mean with 95% confidence interval and numbers in the group in parentheses. Differences between the groups were analysed by two-way ANOVA with Tukey's multiple comparison post-test. Statistical significance is shown for comparisons with group A1 (shown in bold). * $p < 0.05$, ** $p < 0.01$.

		Anti-dsDNA level IU/L		
		Min: 0 to 50 Max: > 50 **1**	Min: 0 to 50 Max: 0 to 50 **2**	Min: >100 Max: >200 **3**
Age at diagnosis	High Trm **A**	21 (-106-148; 2)	**38.4** (25.8-51.0; 5)	27.0 (14.3-39.7; 4)
	High Tem **B**	19.4* (15.0-23.9; 16)	29.6 (23.2-36.1; 8)	21.7* (14.1-29.3; 15)
	Low Tem **C**	26.5 (-132-185; 2)	23.7 (19.1-28.3; 7)	22.5 (16.3-28.7; 4)

Table 5.6.4 – Age at diagnosis of SLE patients treated with BCDT
Age shown in groups of patients that are divided according to their memory T cell phenotypes (rows; Low Tem (N), High Tem with low Trm (E) or High Trm (R)) and how anti-dsDNA antibody levels change after BCDT (columns; High at baseline (>50 IU/L) and fall after BCDT (1), Low (2) or Persistently elevated (3)). Data shows the mean with 95% confidence interval and numbers in the group in parentheses. Differences between the groups were analysed by two-way ANOVA with Tukey's multiple comparison post-test. Statistical significance is shown for comparisons with group A2 (shown in bold). * $p < 0.05$.

		Anti-dsDNA level IU/L		
		Min: 0 to 50 Max: > 50 **1**	Min: 0 to 50 Max: 0 to 50 **2**	Min: >100 Max: >200 **3**
Median disease free survival following BCDT (weeks)	High Trm **A**	48 (n=3)	41 (n=5)	8 (n=3)
	High Tem **B**	89.5 (n=16)	56 (n=6)	40 (n=15)
	Low Tem **C**	287 (n=2)	150 (n=7)	295.5 (n=4)

p=0.02

Table 5.6.5 – Disease free survival of SLE patients following BCDT
Disease free survival shown in groups of patients that are divided according to their memory T cell phenotypes (rows; Low Tem (N), High Tem with low Trm (E) or High Trm (R)) and how anti-dsDNA antibody levels change after BCDT (columns; High at baseline (>50 IU/L) and fall after BCDT (1), Low (2) or Persistently elevated (3)). Data shows the median time to relapse with numbers in each group in parentheses. Differences between the groups were analysed by log-rank (Mantel-Cox) test.

5.6.3 Discussion

In chapter 5.5 it was suggested that SLPCs are formed by ELT, which are initiated by the migration and then aggregation of T cells in the non-lymphoid tissue. The results of this study support this model by showing that patients with low anti-dsDNA antibody levels at remission that then increase during relapse have higher percentages of $CD4^+$ T cells that express chemokine receptors for the non-lymphoid tissue and vascular adhesion molecules. The markers CXCR3 and CCR5 have been seen on $CD4^+$ T cells in renal tissue and urine from patients with nephritis, whilst CD49d has been seen on $CD4^+$ T cells of patients with vasculitis but this is the first study to suggest that these markers might be important for the development of ELT and the formation of SLPCs.

This study also shows that patients with anti-dsDNA antibody levels that do not return to normal after B cell depletion have high percentages of $CD4^+CCR7^+CD62L^+$ T cells that migrate to SLOs, supporting murine studies that show that LLPCs are generated in the SLOs. The results also provide evidence to suggest that LLPCs are formed by T cell help, which has not been shown before in human SLE.

The pathogenesis of SLE therefore appears to be characterized by two distinct processes – the first is an effector memory immune response initiated by T cell activation and migration, followed by ELT formation leading to SLPCs (Figure 5.6.2). The second is a central memory immune response defined by circulating anti-dsDNA antibodies produced by LLPCs generated and maintained in SLOs (Figure 5.6.3). Both processes are likely to play a role in the disease, although to varying degrees in different patients, and overlap in some patients. As a result patients would be expected to have different responses to therapy, in particular BCDT, which is supported by the observed differences in the median time to relapse.

Patients with mainly cellular inflammation will have disease that responds relatively rapidly to B cell depletion but relapse as soon as B cells repopulate, whilst patients with mostly antibody mediated pathology might take longer to respond, depending on how long immune

complexes are cleared from the circulation and tissue and the time it takes for the LLPCs to eventually die but they then appear to take longer to relapse. However, some patients (in group A3 in particular) appear to have features of both cellular inflammation (with moderately high percentages of $CD4^+CCR5^+$ T cells and Trm cells) and LLPCs (with moderately high percentages of $CD4^+CCR7^+CD62L^+$ T cells), which is the most likely explanation for their poor response to BCDT. On the other hand patients with low percentages of $CD4^+CCR5^+$ T cells, SLPCs, low anti-dsDNA antibody levels at baseline and low percentages of memory T cells (groups C1 and C2) have the best responses to BCDT as would be predicted.

The good response to BCDT by patients in group C3, who have persistently elevated anti-dsDNA antibody levels is surprising and difficult to explain. All of the patients in this group had renal disease, but it is not clear why depleting B cells would lead to an improvement in disease, particularly renal disease, if anti-dsDNA antibody levels remain high. It is possible that the renal lesions are due to other nephritotoxic antibodies, such as anti-nucleosome antibodies, that can decrease after BCDT (435) or that B cells are needed for an additional reason that has not yet been elucidated. Anti-dsDNA antibodies cause variable proteinuria in SCID mice (194), supporting the possibility that B cells might be an additional requirement for glomerular inflammation. Chapter 5.4 showed that the skin histology of patients with high anti-dsDNA antibodies and cutaneous disease had small perivascular T cell infiltrates. These T cell infiltrates might be important for allowing antibodies to cross the endothelium, as has been demonstrated in autoimmune neuropathies (445, 446). It is possible that B cells are also important in activating these perivascular T cells, although this mechanism does not appear to be associated with HLA-DR expression by $CD4^+CD49d^{hi}$ T cells.

One study has suggested that there are two different types of renal disease in SLE - one that is dependent on circulating immune complexes and another that is dependent on lymphocytic infiltration (152). This hypothesis is supported by the results from this study. It would be interesting to compare the renal lesions between patients in groups A1 and B1 with those in

B3 and C3. I would hypothesize that patients in group A1 and B1 would have more interstitial infiltrates and vasculitis, whilst patients in B3 and C3 would have more pure glomerular lesions with fewer lymphocytic infiltrates.

The mechanisms that induce T cells to differentiate into either central or effector memory in SLE are unknown but is likely to be due to a combination of cytokines, including IFN-α and IL-6, secreted by APCs that have been stimulated by nuclear antigen. Each group had slightly different patterns of ENA antibodies (data not shown). Group A1 had Ro antibodies only, whilst the patients in the C3 group all had Sm and RNP antibodies. It is tempting to speculate that these different combinations of autoantibodies might be inducing the secretion of a different combination of cytokines, although it is also possible that they reflect the differences in the pathogenesis between a more cellular immune process and a humoral immune process.

This study has so far shown that the four factors that appear to predict the rate of disease flare after BCDT are: 1) the rate of B cell repopulation, 2) B cell differentiation to the $IgD^{-}CD27^{-}$ memory subset, 3) anti-dsDNA antibody levels at the time of B cell repopulation, which is associated with the tendency of $CD4^{+}$ T cells to migrate to SLOs where LLPCs are likely to be produced and 4) the ability of $CD4^{+}$ T cells to form ELT to produce SLPCs that can make more anti-dsDNA antibodies, which is associated with the tendency of $CD4^{+}$ T cells to migrate to the non-lymphoid tissue. A method of stratifying patients into nine groups is proposed using markers that are associated with differences in the rates of B cell repopulation and differentiation (memory T cell subsets) and chemokine receptors for either non-lymphoid tissue and/or SLOs (Table 5.6.6). As expected group A3, that had all four of these factors (high percentage of Trm cells that are associated with fast rates of B cell repopulation and high percentages of $IgD^{-}CD27^{-}$ memory B cells), and $CD4^{+}$ T cells that expressed chemokine receptors for SLOs and the non-lymphoid tissue and therefore had the poorest response to BCDT. This group possibly corresponds to the group that we have previously identified in an open-label study that does not respond to BCDT (425).

This is a unique method of stratifying patients and comparing clinical parameters and outcomes to therapy. It has three important advantages. The first is that it provides an opportunity to identify combinations of factors that might contribute towards the pathogenesis of the disease symptoms and manifestations, and as a result identify the biomarkers and therapeutic targets that might be helpful for that group of patients. For example, vasculitis was most common in a group of patients with high levels of CD49d. However, it was not seen in group A1 where CD49d was also high, suggesting that an additional factor, possibly CD11a, is also required (164). NPLE was more common in patients with high percentages of Trm cells suggesting that a combination of factors such as $IgD^{-}CD27^{-}$ B cells, and the expression of CCR5 and possibly CXCR3 by $CD4^{+}$ T cells might be important, supported by a study showing high CSF levels for chemokines that are specific for both of these chemokine receptors (551). Lymphadenopathy might be due to a combination of CXCR3 and CCR7 expression by $CD4^{+}$ T cells and anti-dsDNA antibodies. However, this analytical approach might have certain limitations. For example, the lack of a significant difference in symptoms such as serositis, alopecia and skin inflammation between the groups might either suggest that T cells do not have an important role in the pathogenesis or that a chemokine receptor that is expressed in high percentages by $CD4^{+}$ T cells in all or most groups, such as CCR4, might contribute to the disease. Skin lesions from patients with cutaneous LE have been shown to have significant numbers of $CCR4^{+}$ T cells, particularly from scarring lesions (322), but I was unable to detect a specific association between CCR4 and skin disease. One possible explanation is that the clinical data available to me was modest and did not differentiate between the various subtypes of skin disease.

The second and most important potential benefit of this stratification is that it might help to predict the likely outcome from therapeutic interventions or expect different responses to therapy. A larger prospective study would be needed to validate this method of stratification but if confirmed future clinical trials should achieve more predictable and positive outcomes.

The third benefit is that once patients are stratified it becomes possible to then quantify the progression of the disease, particularly in the patients with low anti-dsDNA antibody levels at remission (explained in discussion 5.5.3). Some of these biomarkers are not useful for patients with anti-dsDNA antibody levels that remain high during remission – i.e. $IgD^{-}CD27^{-}$ B cells and $CD4^{+}CD49d^{hi}HLA\text{-}DR^{+}$ T cells. However, this group can be identified by the high expression of CCR7 and CD62L by $CD4^{+}$ T cells (Figures 5.6.1 and 5.6.3). In this group relapse appears to be associated with the appearance of plasmablasts and possibly a fall in circulating lymphocyte numbers in some patients, although the precise sequence of events that lead to tissue inflammation and the significance of the lymphopaenia is less clear. A better understanding of the disease process in this group with persistently elevated anti-dsDNA antibody levels might result in better biomarkers. Table 5.4.1 in chapter 5.4 showed that the inflamed skin of the patients with high anti-dsDNA antibody levels during active disease had immune deposits with small perivascular T cell infiltrates and low circulating lymphocyte numbers. In this group it is possible that the perivascular T cells cause disruption of the endothelial barrier allowing antibodies and complement to cross into the interstitium.

The complex inflammatory pathway that is illustrated is a proposal. An *in vivo* model would be needed to prove it conclusively, but this would be impossible to do in humans and animal models have obvious limitations, such as being too homogeneous. However, the model predicts that the relapse rate of patients after BCDT will be influenced by T cell differentiation and their chemokine receptors, which has proven correct, and therefore supports the proposed model of disease.

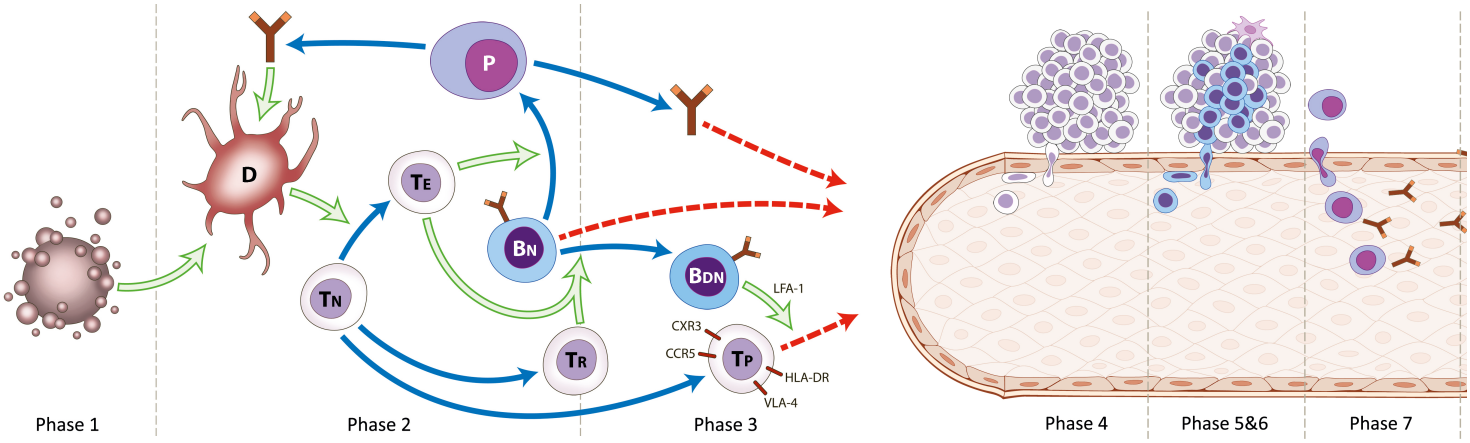


Figure 5.6.2 – Development of short-lived plasma cells in ectopic lymphoid tissue
The development of short-lived plasma cells that produce anti-dsDNA antibodies is associated with the expression of CXR3, CCR5 and CD49d (VLA-4) by $CD4^+$ T cells in patients with high percentages of $CD4^+CD45RA^+CD27^-$ revertant T cells (T_R) suggesting that SLPCs are formed in ectopic lymphoid tissue.

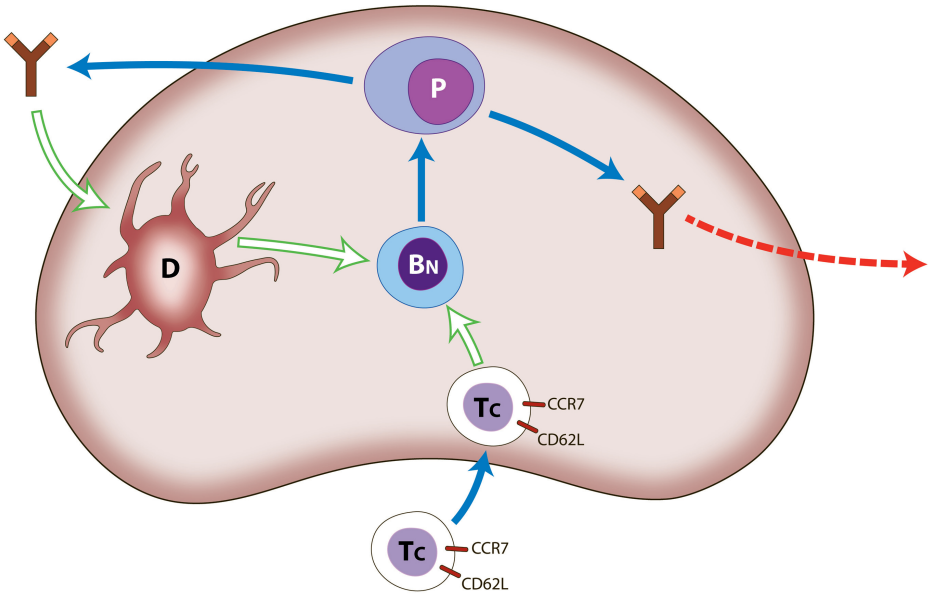


Figure 5.6.3 – Development of long-lived plasma cells in secondary lymphoid organs
The development of long-lived plasma cells that produce anti-dsDNA antibodies is associated with the expression of CCR7 and CD62L by $CD4^+$ T cells in patients with low percentages of $CD4^+CD45RA^+CD27^-$ revertant T cells (T_R) suggesting that LLPCs are formed in germinal centres in the SLOs, such as the lymph nodes, spleen and bone marrow. Recruitment of myeloid dendritic cells into the germinal centres in the SLOs is also likely to be important in this process.

High Trm Low to Moderate DNA CXCR3 ++++ CCR5+++ CD49d +++ CD62L/CCR7 – Arthritis/Nephritis/Cutanous/ NPLE	High Trm Low DNA CXCR3 +++ CCR5++ CD49d + CD62L/CCR7 – Arthritis/Mucocutanous/NPLE	High Trm High DNA CXCR3 ++ CCR5++ CD49d ++ CD62L/CCR7 + Arthritis/Nephritis/ Mucocutanous/NPLE/ Lymphadenopathy
High Tem/ Low Trm Low to High DNA CXCR3 ++ CCR5++ CD49d ++ CD62L/CCR7 + **Vasculitis/**Nephritis/ Mucocutanous	High Tem/ Low Trm Low DNA CXCR3 ++ CCR5++ CD49d + CD62L/CCR7 + **NPLE/ Mucocutaneous/** Nephritis	High Tem/ Low Trm High DNA CXCR3 ++ CCR5+ CD49d + CD62L/CCR7 ++ **Nephritis/Mucocutanous/** Lymphadenopathy
Low Tem Low to High DNA CXCR3 + CCR5- CD49d + CD62L/CCR7 +++ **Vasculitis/Cutaneous**	Low Tem Low DNA CXCR3 + CCR5- CD49d + CD62L/CCR7 ++ **Cutaneous/**Arthritis/Nephritis	Low Tem High DNA CXCR3 + CCR5+ CD49d + CD62L/CCR7 ++ **Nephritis**

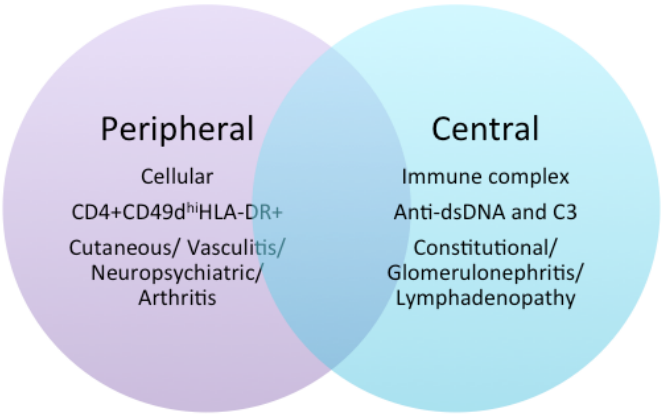


Table 5.6.6 – Summary of groups according to T cell markers and anti-dsDNA antibody levels

5.7 The monocyte recruiting chemokines, IP-10 and MCP-1, are elevated in SLE patients with high anti-dsDNA antibody levels that do not fall after BCDT

5.7.1 Introduction

Chemokines recruit leukocytes to SLOs and sites of inflammation. The serum, tissue and urinary levels of several chemokines are increased in patients with active SLE (316, 322, 388, 412, 413, 551-553), but the precise source of the chemokines and their effect on leukocytes in the disease is unclear. One study has suggested that DCs are the main source of chemokines in inflamed tissue (554). However, lymphocytes are seen in non-lymphoid tissue even when DCs are not present (152, 153), suggesting that they might be another important source of the chemokines.

B cells are able to produce several different chemokines (50, 183, 416). Therefore, it was hypothesized that they are the source of the high levels of chemokines in the serum of patients with SLE and that B cell depletion would lead to a fall in the serum levels of the chemokines that attract T cells and APCs either to SLOs, where LLPCs are formed, or to non-lymphoid tissue.

Some of the results in this study were obtained by Andrew Fester for his intercalated BSc project, which I co-supervised.

5.7.2 Results

MCP-1 and IP-10 levels positively correlate with anti-dsDNA antibody levels before BCDT

Immune complexes containing anti-dsDNA antibodies have been shown to increase the synthesis of chemokines by both DCs and B cells (183). Several of these chemokines have been shown to be increased in the serum or urine of patients with active SLE (413) and attract T cells (RANTES, MIG, MCP-1 and IP-10), monocytes (MCP-1, IP-10, IL-8) and neutrophils (IL-8). I therefore examined whether there was a correlation between anti-dsDNA antibody levels and the serum levels of the chemokines MCP-1, IP-10, MIG, RANTES and IL-8 in SLE patients not previously treated with rituximab (n = 23). The results showed that there was a positive correlation between anti-dsDNA antibody levels and MCP-1 ($r_p = 0.52$, $p = 0.014$) and IP-10 levels ($r_p = 0.54$, $p = 0.009$) but there was no correlation with RANTES ($r_p = 0.31$, $p = 0.15$), MIG ($r_p = –0.05$, $p = 0.80$) or IL-8 levels ($r_p = 0.10$, $p = 0.67$) (Figure 5.7.1). These results are supported by another study, which has also shown a positive correlation between IP-10 and anti-dsDNA antibody levels (553).

MCP-1 and IP-10 levels are not affected by the presence of B cells but might be increased by high anti-dsDNA antibody levels

It was then hypothesized that if anti-dsDNA antibodies induce the synthesis of MCP-1 and IP-10 then the serum levels of these chemokines would only fall after BCDT in the group of patients whose anti-dsDNA antibody levels also fall. As in the previous study patients were divided into 3 groups; 1) low (<50 IU/L; n = 8), 2) fluctuating (>50 IU/L at baseline but fall by 50% and below 100 IU/L after BCDT; n = 5) and 3) persistently high anti-dsDNA antibody levels (>100 IU/L following BCDT; n = 5). The results showed that MCP-1, IP-10 and MIG levels pre-BCDT were higher in patients with high anti-dsDNA antibody levels that did not fall after BCDT (group 3) compared to patients with low anti-dsDNA antibody levels (group 1) ($p < 0.0001$ for MCP-1, $p < 0.001$ for IP-10 and $p < 0.05$ for MIG, 2-way ANOVA with Sidak's multiple comparisons test) and compared to patients with high anti-dsDNA

antibody levels that fell after BCDT (group 2) ($p < 0.0001$ for MCP-1, $p < 0.01$ for IP-10 and $p < 0.05$ for MIG, 2-way ANOVA with Sidak's multiple comparisons test) (Figure 5.7.2). MCP-1 and IP-10 levels post BCDT were also higher in patients with high anti-dsDNA antibody levels that did not fall after BCDT (group 3) compared to patients with low anti-dsDNA antibody levels (group 1) ($p < 0.0001$ for MCP-1 and $p < 0.01$ for IP-10, 2-way ANOVA with Sidak's multiple comparisons test) and compared to patients with high anti-dsDNA antibody levels that fell after BCDT (group 2) ($p < 0.0001$ for MCP-1 and $p < 0.01$ for IP-10, 2-way ANOVA with Sidak's multiple comparisons test). IL-8 levels also were higher in group 3 compared to group 1 following BCDT ($p < 0.05$, 2-way ANOVA with Sidak's multiple comparisons test).

After BCDT none of the chemokine levels changed significantly in the groups of patients with low anti-dsDNA antibody levels pre-BCDT (group 1) or in the group of patients with high anti-dsDNA antibody levels that did not change after BCDT (group 3). However, in the group of patients whose anti-dsDNA antibody levels were high pre-BCDT and fell after BCDT (group 2) there was a significant fall in IL-8 levels ($p = 0.04$ for IL-8, paired t test), although the baseline levels were not significantly higher than the levels in patients with low anti-dsDNA antibody levels (group 1). There were no significant changes in MCP-1, IP-10, MIG and RANTES levels after BCDT. These data therefore support the studies that show that anti-dsDNA antibodies induce the synthesis of MCP-1, IP-10 and IL-8. B cells might produce IL-8 or induce its production by other cells, but they do not appear to be important for the production of MCP-1, IP-10, MIG and RANTES.

BCR stimulated PBMCs produce higher levels of MCP-1 and IL-8 but purified BCR stimulated B cells only produce IL-8

Studies have shown that B cells can produce MCP-1 (202). To examine whether B cells from SLE patients produce higher levels of MCP-1, PBMCs from healthy controls (n = 3) and SLE patients (n = 7) were cultured with goat anti-human Ig $F(ab')_2$, which activates B cells by binding to the BCR. The results showed that SLE PBMCs produced higher levels of MCP-1

and IL-8 when stimulated (p = 0.031 and 0.046 respectively, Wilcoxon matched-pairs signed rank test) (Figure 5.7.3). Healthy PBMCs also appeared to produce higher levels of MCP-1 and IL-8 but the numbers of samples used was too small to reach significance. There were no significant differences in the levels of IP-10, MIG or RANTES when healthy or SLE PBMCs were stimulated.

To determine whether B cells were producing MCP-1 and IL-8, B cells were purified from the PBMCs from healthy controls (n = 3) and SLE patients (n = 8) and cultured with goat anti-human Ig $F(ab')_2$. Stimulated B cells from SLE patients produced higher levels of IL-8 (p = 0.016, Wilcoxon matched-pairs signed rank test) but did not produce higher levels of MCP-1, IP-10, MIG or RANTES (Figure 5.7.4). The observation that stimulated B cells from SLE patients produce IL-8 is consistent with a study showing B cell production of IL-8 following stimulation with immune complexes containing DNA (183). The data therefore suggest that B cells synthesize IL-8 and induce the synthesis of MCP-1 via other cells, most likely myeloid cells.

Monocyte levels do not rise after BCDT in patients with persistently elevated anti-dsDNA antibody levels

One study has shown that monocyte levels increase after BCDT. It was hypothesized that if MCP-1 and IP-10 levels remain elevated after BCDT in patients with persistently elevated anti-dsDNA antibody levels then monocyte numbers should also remain low in this group. The monocyte counts of patients with low (group 1; n = 11), fluctuating (group 2; n = 11) and persistently elevated anti-dsDNA antibody levels (group 3; n = 16) who relapsed when B cells repopulated after BCDT were therefore compared (Figure 5.7.5). The results showed that the mean monocyte number of patients in group 3 was 0.35 x 10^9 cells/L at baseline (normal range 0.2 to 1.0 x 10^9 cells/L) compared to 0.49 x 10^9 cells/L in group 1 and 0.50 x 10^9 cells/L in group 2, although it was not significantly lower than the other groups. However, 5/16 patients had a monocyte count below the normal range compared to 2/11 patients in group 1 and 1/11 in group 2 with fluctuating anti-dsDNA antibody levels. There were no significant

changes in monocyte numbers between baseline and B cell depletion in any of the groups. Surprisingly, monocyte numbers fell as B cells repopulated in group 1 ($p < 0.01$, Wilcoxon matched-pairs signed rank test), but there were no significant changes in monocyte numbers after B cells repopulated in groups 2 and 3 with high anti-dsDNA antibody levels, suggesting that B cells are influencing monocyte numbers independently of anti-dsDNA antibodies. It is possible that IL-8 influences circulating monocyte numbers as it was previously shown that B cells can produce IL-8 with just BCR stimulation.

$CD14^+$ cells were not seen in high numbers in the biopsies of patients with cutaneous disease

To examine whether monocytes might be present in the inflamed tissue, skin biopsies from the active lesions of five patients were stained for CD14. The results showed that $CD14^+$ myeloid cells were seen in small numbers (5 to 10% of cells) in three of the five biopsies, from patients 78, F and G (Table 5.7.1). There was no relationship between circulating monocyte numbers and the presence of monocytes in the skin. Thus, the cause of the low circulating monocyte numbers in patients with SLE remains unclear.

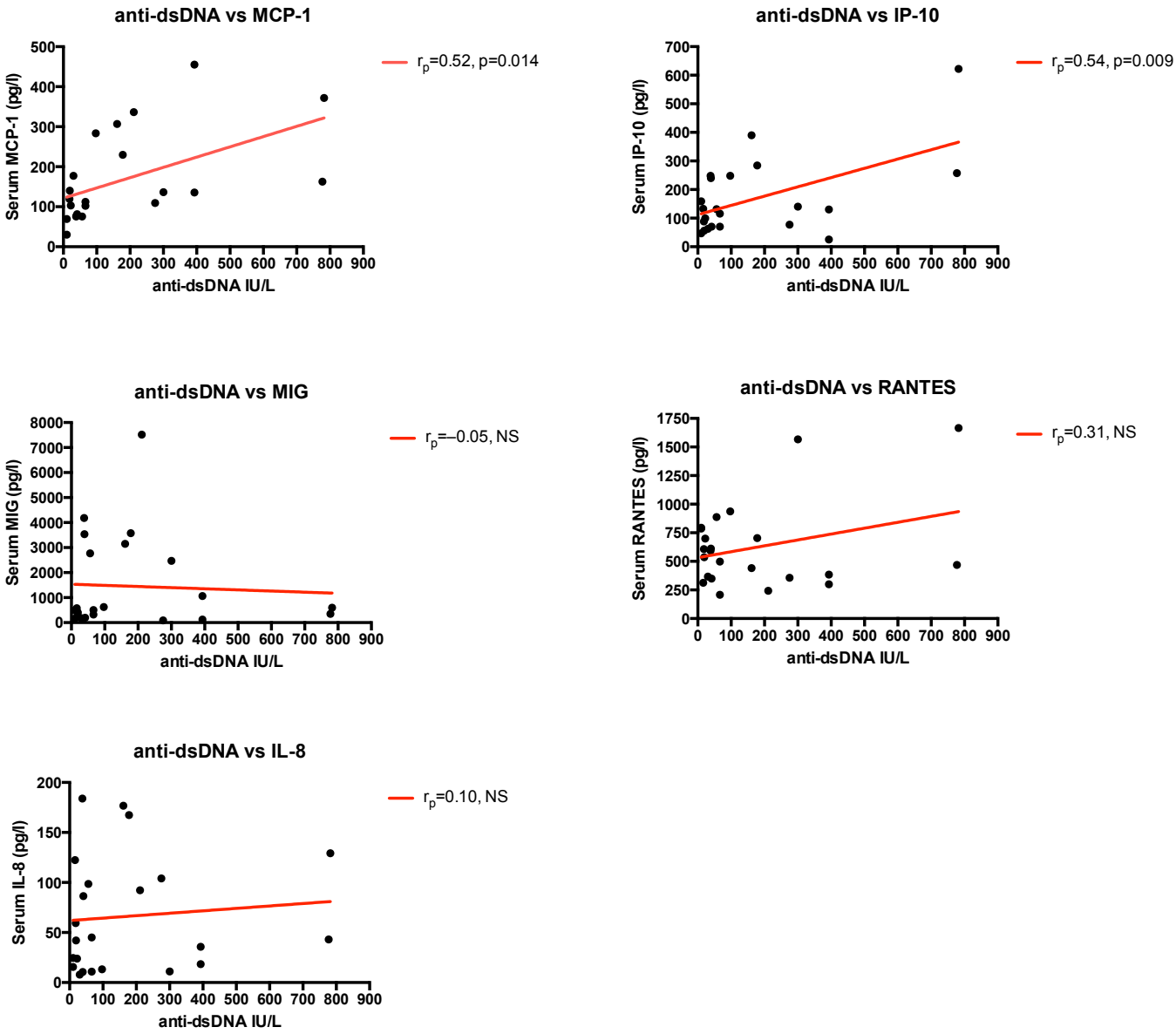


Figure 5.7.1 – Relationship between the serum levels of the chemokines MCP-1, IP-10, MIG, RANTES and IL-8 and anti-dsDNA antibody levels in patients with SLE not treated with BCDT

Serum chemokine levels are plotted against the anti-dsDNA antibody levels of patients with SLE not treated with BCDT [n = 23]. Data are represented as scatter plots with lines of best fit. The Pearson correlation coefficient (r_p) is provided for each association. NS = $p > 0.05$.

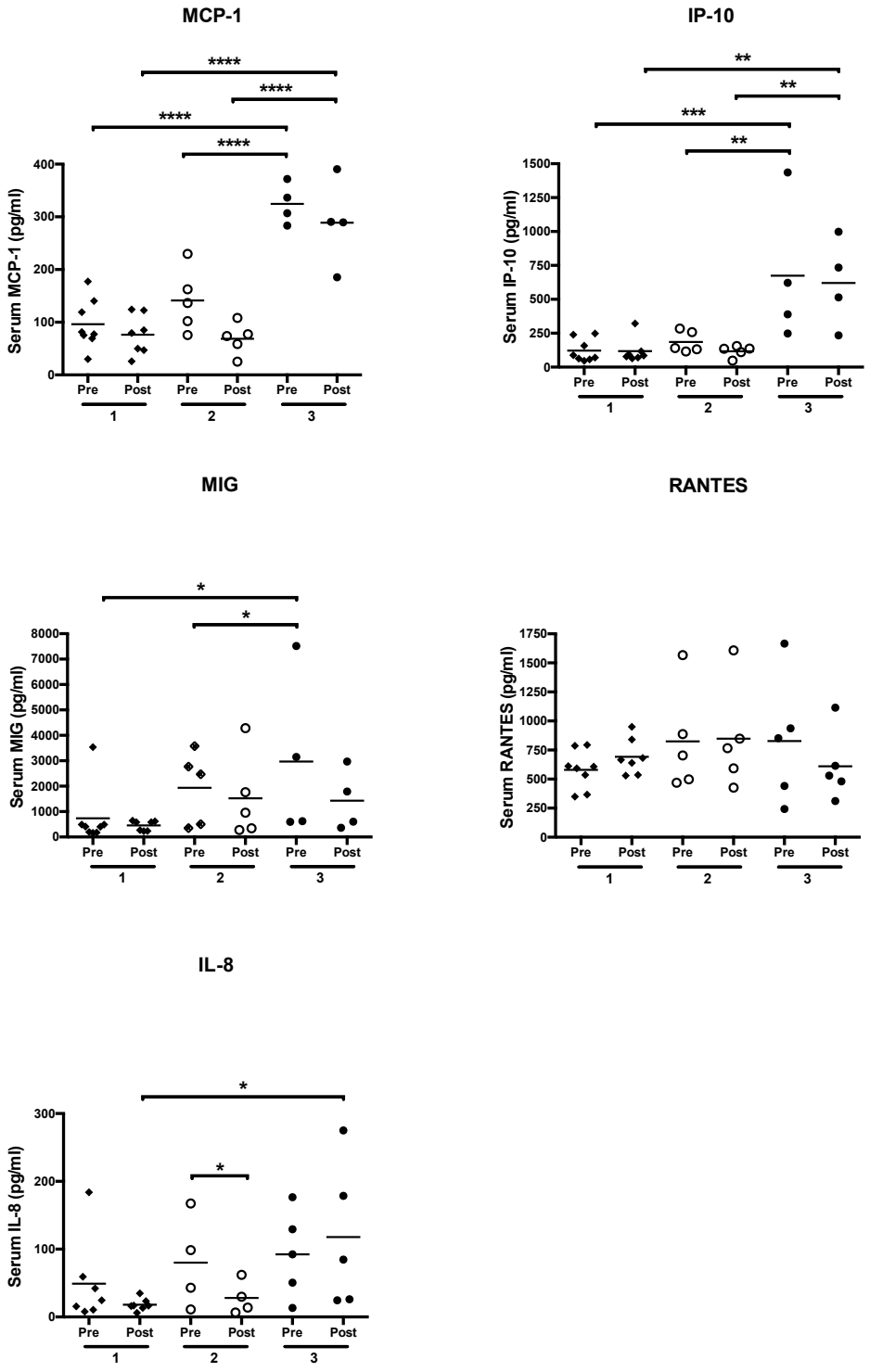


Figure 5.7.2 – Serum levels of chemokines MCP-1, IP-10, MIG, RANTES and IL-8 in SLE patients with active disease treated with BCDT

Serum levels of chemokines from SLE patients treated with BCDT that have been divided into three groups according to how their anti-dsDNA antibody levels change after treatment; Group 1 – low anti-dsDNA antibody levels [<50 IU/L; n = 8], Group 2 – high anti-dsDNA antibody levels that fall after BCDT [>50 IU/L and fall by 50% and below 100 IU/L; n = 5] and Group 3 – high anti-dsDNA antibody levels that do not fall after BCDT [>100 IU/L; n = 5]. Data are shown as dot plots with lines representing the mean. Differences between groups were analysed by 2-way ANOVA and paired t test for paired data. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

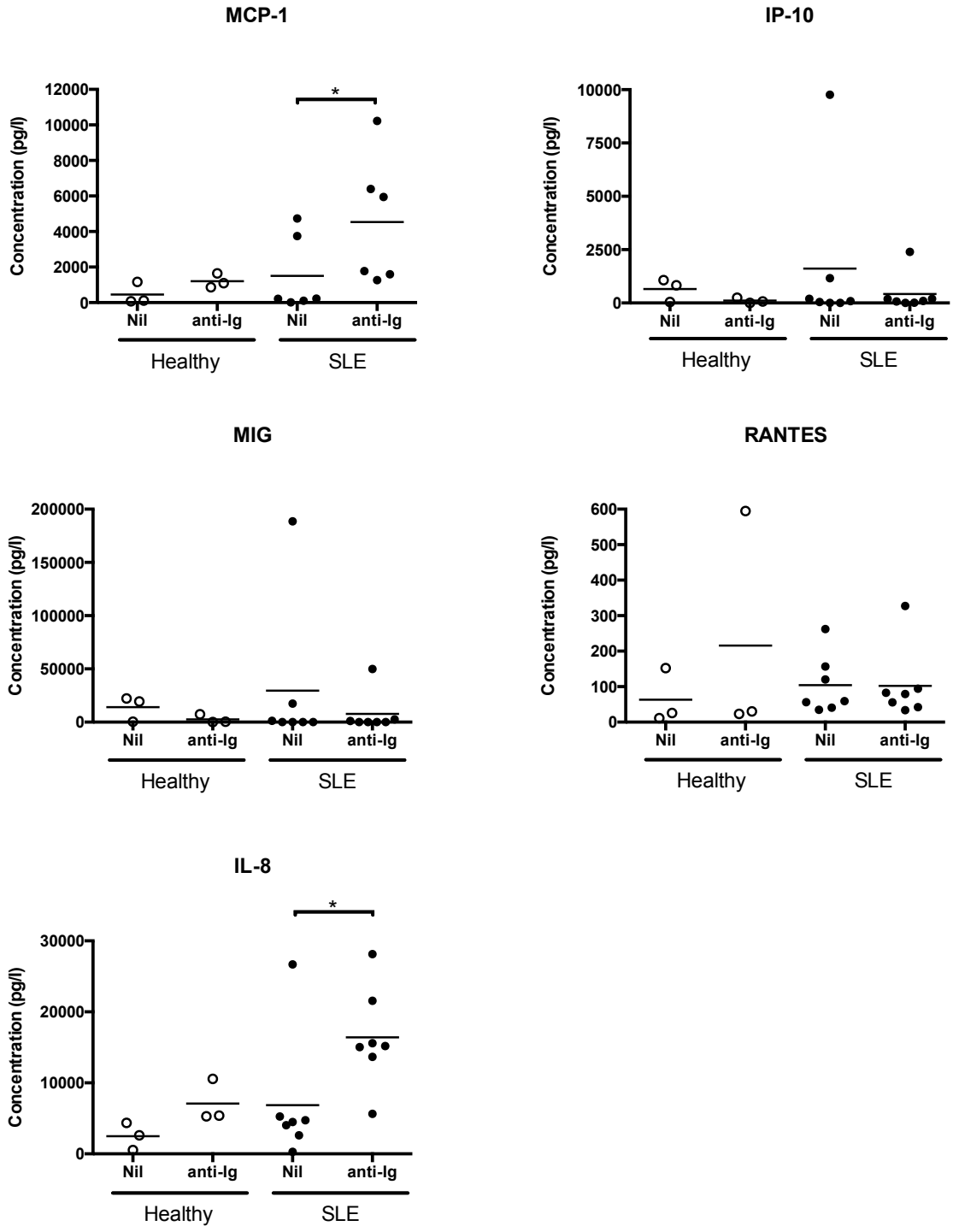


Figure 5.7.3 – Expression of chemokines MCP-1, IP-10, MIG, RANTES and IL-8 by PBMCs following BCR stimulation *in vitro*

PBMCs were obtained from healthy individuals [n = 3] and patients with SLE [n = 7]. 2.5 x 10^6 cells were cultured in 200 μl of complete medium (RPMI with 10% foetal calf serum) for 48 hours with either no stimulant (Nil) or 2.5 μg/ml $F(ab')_2$ anti-Ig (anti-Ig). Chemokines were measured by cytometric bead array. Data are shown as dot plots with lines representing the mean. Differences between groups were analysed by Mann-Whitney U test for unpaired data and Wilcoxon matched-pairs signed rank test for paired data. * $p < 0.05$.

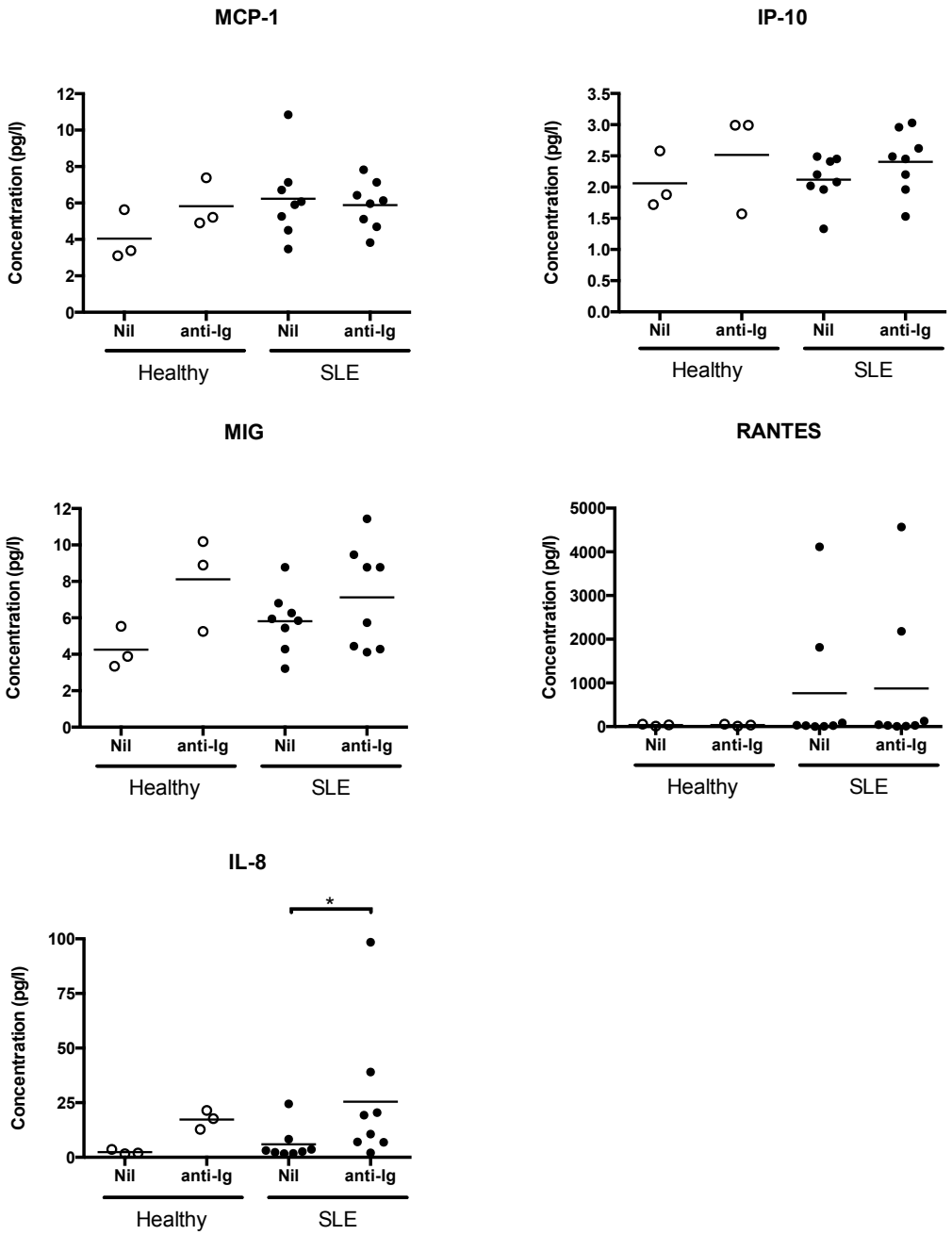


Figure 5.7.4 – Expression of chemokines MCP-1, IP-10, MIG, RANTES and IL-8 by B cells following BCR stimulation *in vitro*

PBMCs were obtained from healthy individuals [n = 3] and patients with SLE [n = 8]. B cells were isolated by incubating the PBMCs with a cocktail of antibodies against CD2, CD14, CD16, CD36, CD43, and CD235a attached to MicroBeads and passing the cells through magnetic columns. 2.5 x 10^6 cells were cultured in 200μl of complete medium (RPMI with 10% foetal calf serum) for 48 hours with either no stimulant (Nil) or 2.5 μg/ml $F(ab')_2$ anti-Ig (anti-Ig). Chemokines were measured by cytometric bead array. Data are shown as dot plots with lines representing the mean. Differences between groups were analysed by Mann-Whitney U test for unpaired data and Wilcoxon matched-pairs signed rank test for paired data. * $p < 0.05$.

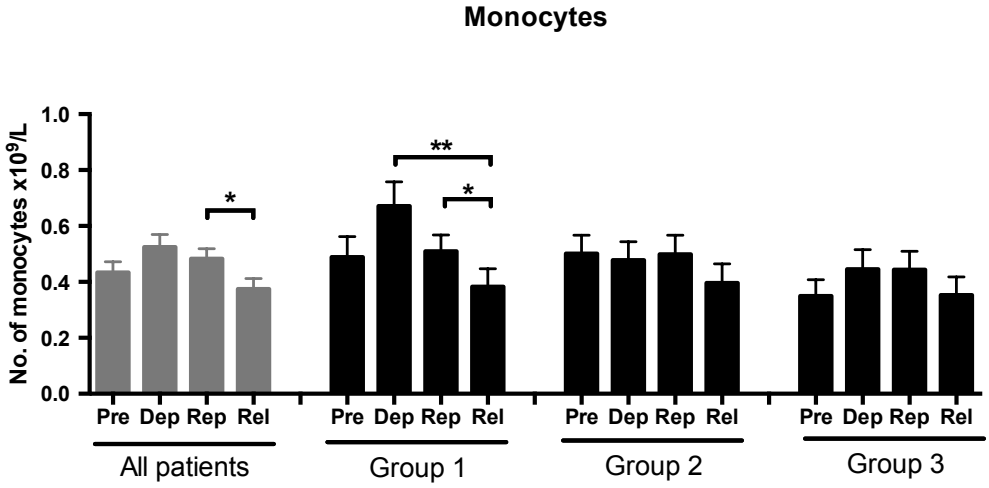


Figure 5.7.5 – Changes in monocyte numbers following BCDT in relapsing patients
Monocyte numbers are shown at baseline, during B cell depletion, at B cell repopulation and at remission (6 months post repopulation) or at clinical relapse in all patients [n = 38] and in three groups that have been divided according to how their anti-dsDNA antibody levels change after treatment; Group 1 – low anti-dsDNA antibody levels [<50 IU/L; n = 11], Group 2 – high anti-dsDNA antibody levels that fall after BCDT [>50 IU/L and fall by 50% and below 100 IU/L; n = 11] and Group 3 – high anti-dsDNA antibody levels that do not fall after BCDT [>100 IU/L; n = 16]. Columns indicate the means; bars indicate the S.E.M. Differences between the groups were analysed by Wilcoxon matched-pairs signed rank test for paired data. * $p < 0.05$, ** $p < 0.01$.

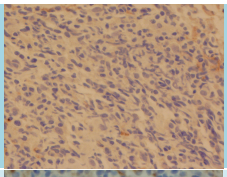
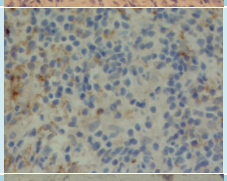
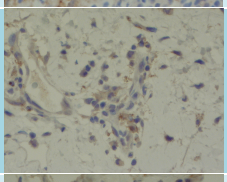
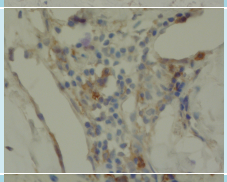
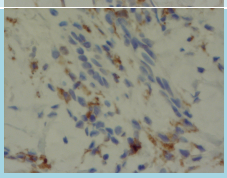
ID	CD14⁺ cells	IMF	Disease duration	Symptoms	ENA	Anti-dsDNA (IU/L)	C3 (g/dL)	Mono ($x10^9$/L)
62		Granular deposits of IgM and C3 at the BMZ. In addition there is epidermal ANA staining.	7 months (2008)	Malar rash, fatigue and arthralgia	Ro+ La+	11	0.83	0.52
78		Data not available	11 years (1999)	Malar rash only. No constitutional symptoms (Digital vasculitis and fever after 9 months)	RNP+ Sm(+) Ro(+)	62 (2686 after 9 months)	1.07 (0.30 after 9 months)	0.37 (0.20 after 9 months)
54		Speckled BMZ staining with IgM, IgA and to a lesser extent with C3.	22 years (1985)	Diffuse desquamating rash, serositis and fever	Negative	70	0.60	0.10
F		Granular deposits of IgM, IgG, IgA and C3 at the BMZ.	7 years (2002)	Diffuse macular rash, alopecia, mouth ulcer, nephritis (class II), arthralgia, and fever	Ro+	366	0.39	0.24
G		Granular deposits of IgM, IgG, IgA at the BMZ and colloid bodies labelling with IgM, IgG and IgA in the papillary dermis and along the dermo-epidermal junction.	1 month (2010)	Diffuse desquamating rash, hair loss, thrombocytopaenia, arthralgia, weight loss and fever	Negative	300	0.38	0.11

Table 5.7.1 – Comparison between myeloid cell infiltrates in lupus skin lesions and anti-dsDNA antibody and C3 levels before BCDT

Immunohistochemical analysis of myeloid ($CD14^+$) cells in tissue biopsies of active skin lesions from five patients with SLE who had not been treated with rituximab (ID numbers 54, 62, 78, F and G). Original magnification: x400. Results from the direct immunofluorescence are also given with symptomatology, disease duration, ENA antibody seropositivity, anti-dsDNA antibody levels, C3 levels and monocyte numbers.

5.7.3 Discussion

Monocytopaenia is a recognized feature of active SLE (337), which, like lymphopaenia, has also been attributed to the presence of cytotoxic antibodies (555). In chapters 5.4 to 5.6 it was suggested that lymphopaenia is instead due to T cell migration and I think that monocytopaenia is also likely to be due to monocyte migration, either to the non-lymphoid tissue where it contributes to the formation of ELT and/or to SLOs.

Myeloid cells were present in three of the five histological specimens that were examined and studies of renal biopsies also show the presence of monocytes and macrophages near the glomerulus in patients with lupus nephritis showing that monocytes are certainly migrating to the tissue, although possibly only in small numbers. Monocyte migration to SLOs is difficult to demonstrate in humans, but it has been demonstrated in animal models of autoimmune disease (556).

The mechanism of monocytopenia appears to differ between patients. In patients with persistently high anti-dsDNA antibodies the monocytopenia might be related to high serum levels of MCP-1 and IP-10, synthesized by non-B cells. In the previous study it was shown that $CD4^+CCR7^+CD62L^+$ T cells, that migrate to SLOs, might be important for the development of LLPCs. The preliminary evidence that MCP-1 and IP-10 remain high in patients with high anti-dsDNA antibody levels after BCDT suggests that monocyte recruitment might also have a role. This is supported by a study using an experimental model for myasthenia gravis that showed that MCP-1 is important for the recruitment of monocytes to the draining lymph node, where it contributes to the initiation of B cell mediated autoimmunity (556). Therefore, recruitment of monocytes to the SLOs, where they become MDCs, might be an additional step, along with the recruitment of $CD4^+CCR7^+CD62L^+$ T cells, towards the establishment of niches from which LLPCs can develop. This would create a vicious circle, which can only be stopped by depleting the LLPCs or blocking MCP-1 and/or IP-10.

Whilst B cells do not appear to directly recruit monocytes, TLR-9 stimulated B cells have been shown to increase IFN-α production by PDCs (557), which in turn activates monocytes (241). It is also intriguing that $IgD^{low}CD27^{-}$ B cells have been shown to regulate the maturation of monocytes and a defect in this interaction was seen in patients with SLE (558).

Figure 5.7.6 shows a proposed model for the recruitment of monocytes and T cells to the lymph node and/or spleen by B cells and monocytes stimulated by nuclear antigen and immune complexes. Future studies would be needed to confirm this model, but if proven MCP-1 would become a novel target for the reduction in the LLPCs that maintain a propensity for disease flare by continuously secreting pathogenic autoantibodies even during clinical remission.

The high levels of MCP-1 and IP-10 in patients with high anti-dsDNA antibody levels, who have an increased incidence of nephritis, suggests that these chemokines are specifically involved in recruiting monocytes and macrophages to the glomerulus. A role for MCP-1 in nephritis is supported by several studies, which show a consistent association between high levels of urinary MCP-1 with active nephritis (559). However, IP-10 has not been consistently associated with renal disease, although interestingly it has been associated with mucocutaneous disease, anaemia and lymphopaenia (553). The association with lymphopaenia is intriguing because IP-10 can also recruit T cells.

Whilst monocytopaenia might not be directly due to B cells in patients with high anti-dsDNA antibody levels, the repopulation of B cells after BCDT is associated with a fall in monocyte levels in patients with low anti-dsDNA antibody levels. This would suggest that they have a direct role in monocyte recruitment in this sub-group of patients. The precise mechanism is unclear, but IL-8, which B cells can secrete (183), is able to attract monocytes in addition to neutrophils and is a candidate for further investigation. It is possible that the fall in monocyte levels during relapse in this group of patients with low anti-dsDNA antibody levels reflects

the recruitment of myeloid cells and granulocytes to the non-lymphoid tissue by low levels of IL-8 secreted by B cells.

The ligands for IP-10 and MCP-1 are also expressed on $CD4^+$ T cells. IP-10 binds to CXCR3, which is primarily expressed by T_H1 cells (560), and MCP-1 binds to CCR2, which is expressed on T_H17 cells (561). The expression of these chemokine receptors on T cells supports the hypothesis that these chemokines can induce a fall in circulating lymphocyte numbers that have been shown to occur during active disease, but also the recruitment of different helper T cell subsets by these chemokines provides another possible mechanism to explain the heterogeneous manifestations that are seen in the disease.

This chapter reiterates the point made previously in this thesis that B cells have multiple functions and are therefore capable of contributing in several ways to a heterogeneous disease, such as SLE. By selecting the groups of patients according to differences in serology it has been possible to identify different factors that might be important for each subgroup and that stratifying patients might help clinicians to carefully select the optimal treatment for their patients.

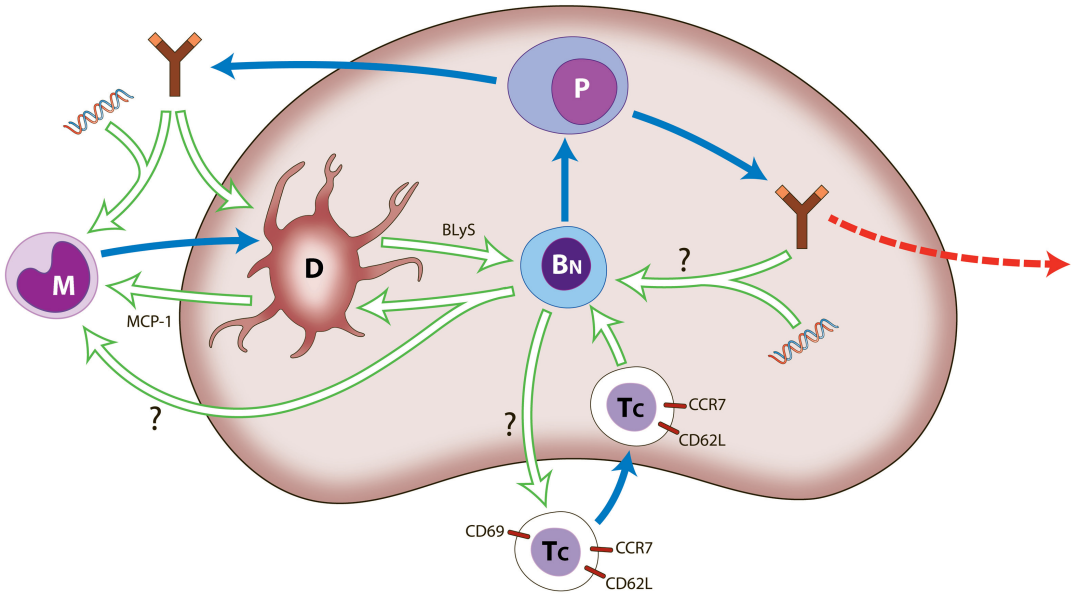


Figure 5.7.6 – A proposed model for the development of long-lived plasma cells in secondary lymphoid organs

B cells induce the development of long-lived plasma cells by recruiting and activating other cells to germinal centres in the SLOs. Myeloid cells are recruited either directly via IL-8, which is produced by BCR or DNA immune complex stimulated B cells, or indirectly by inducing the production of MCP-1 by other myeloid cells. In the case of $CD4^+$ T cells, the mechanism of recruitment might involve inducing CD69 expression.

6 Final Conclusion

Although several human and animal studies have shown that B cells have an important role in the pathogenesis of SLE, RCTs have failed to confirm that depleting B cells from patients is therapeutically beneficial. Most explanations focus on potential problems with the design of the trials, for example possible problems with the disease activity assessment tool that was used and the use of concomitant corticosteroids (146). However, it is important to note that several drugs that target specific molecules or cells have been unsuccessful in RCTs in SLE, unlike drugs with non-specific or broad mechanisms of action (149, 150). RCTs are most likely to be successful if the drug has a broad mechanism of action, if it is interfering in a disease process with either a linear or spiraling causal pattern (i.e. an amplification loop) or if its target is part of an early stage in the disease process that has a domino causal pattern (i.e. a branching process). Therefore, whilst it is possible to conclude from the unsuccessful RCTs that the molecules or cells targeted do not cause SLE it is also possible that the disease process is non-linear and need to be defined better in order to determine how best to use the drugs available and improve the design of RCTs.

There has possibly been an assumption that whilst the symptoms are heterogeneous, B cells have a singular function as autoantibody producing cells in a linear (98) or spiraling disease process (231). In these kind of models, depleting B cells should lead to a very predictable outcome. However, as has been discussed in this thesis, B cells are known to have multiple functions in addition to antibody production and have been shown to have antibody independent roles in an animal model of SLE. It would therefore seem reasonable to suggest that in patients with SLE B cells also have multiple roles that are important at different time-points, and are not part of a simple linear or spiraling process. This thesis has shown that B cells can induce HLA-DR expression by T cells via cognate interactions and that this interaction is most likely part of a different phase to the phase when B cells differentiate into anti-dsDNA antibody producing plasma cells. The B cells that induce HLA-DR expression by

T cells might also be a different subset to the B cells that differentiate into antibody producing plasma cells, which are also likely to differ according to whether they differentiate into short or long-lived plasma cells.

Models of SLE need to show more than just the multiple cellular interactions. They should also show the timeline of the disease by showing the sequence of events from the beginning to the end, i.e. the inflammation in the non-lymphoid tissue, with the time it takes between each event. However, there is significant variation in the factors that initiate events, in particular the genetic factors, termed immune redundancy. There is also variation in the tissue that is affected and in the type of inflammation, although the latter can be broadly divided into four types; lymphocytic infiltration, immune complex deposition, vasculitis and vascular occlusion. Therefore, models also need to show how the immune redundancy can lead to a relatively conserved number of inflammatory events. The most likely explanation is that these aetiological factors result in specific outcomes depending on how and, importantly, where they combine with each other.

The observation that anti-dsDNA antibodies can be produced by either SLPCs or LLPCs shows that there are two pathways that can both produce an apparently similar autoantibody, and is an important example of immune redundancy in SLE. This thesis tries to illustrate how these two pathways appear to be associated with differences in the chemokine receptor expression of $CD4^+$ T cells, suggesting that they are a result of differences in where B and T cells interact in the body. One pathway (Type A) is associated with T cells that migrate to the non-lymphoid tissue, whilst the other pathway (Type B) is associated with T cells that migrate to SLOs (Figure 6.1.1).

There has been a debate in the literature about whether the cause of tissue damage in SLE is due to an Arthus (immune complex mediated) reaction, a Schwartzman (cell mediated cytotoxicity) reaction or an alternative process (162, 163). The data shown in this thesis suggest that type A disease leads to inflammation consistent with the Schwartzman reaction

(vasculitis), whilst type B disease leads to inflammation consistent with the Arthus reaction (i.e. glomerulonephritis). Therefore, both models appear to be correct. However, the likelihood of developing tissue damage due to the Arthus or Schwartzman reaction depends on the expression of chemokine receptors by the T cells and the origin of the autoantibodies (i.e. ELT or SLOs).

T cells have been shown to increase endothelial permeability to antibodies (445-447). In type B disease where and how T cells adhere to the vascular endothelium and then influence endothelial permeability might also account for which tissue autoantibodies can enter to induce damage. The number of T cells required to induce endothelial permeability might vary depending on the tissue. For example, the glomerulus, which is probably more permeable to proteins, might require less T cells than the cerebral endothelium.

The two different pathways are likely to combine to varying degrees, resulting in a spectrum disorder, suggesting that the disease is also partly due to relational causality. Most importantly the differing disease process in each patient, with differing rates of progression, will lead to different times to clinical relapse. The different processes might also result in different times to clinical response, although this was not examined in this thesis.

Different symptoms might also occur at different phases of SLE. For example, fatigue and joint pain might be a symptom of an early phase due to cytokines that contribute to T cell differentiation, such as IFN-α, whilst symptoms due to ELT formation, such as the malar rash, which is a manifestion of ILE that predicts SLE (110, 112), are likely to be a feature of an intermediate phase (in Type A disease). Vasculitis, neuropsychiatric disease, thromboses and disease due to immune complex deposition (e.g glomerulonephritis), which require combinations of factors to occur at the same time, are almost certainly late manifestations of SLE (in both Type A, B and mixed disease). Importantly, vasculitis, renal and neuropsychiatric disease, which are associated with higher incidences of mortality and

morbidity, can potentially be predicted before they occur using the T cell markers of the two different pathways (Table 6.1.1).

The potential difference in the timepoints for when certain symptoms appear show the problems with the current disease activity assessment tools, such as the BILAG index, which do not appear to differentiate between symptoms according to the underlying pathology or where the symptoms might occur in the disease process. Giving glomerulonephritis, which is caused by immune complex deposits, a similar score to discoid lupus, which is caused by lymphocytic infiltrates, regardless of whether symptoms are new or worse is unlikely to reflect the disease process accurately.

The proposed model shows how different combinations of factors could theoretically lead to different symptoms. It also provides an explanation for why there are no biomarkers that directly correlate with active disease. Biomarkers should instead be interpreted in relation to other factors. For example, high levels of anti-dsDNA antibodies might only be significant if other factors that anti-dsDNA antibodies combine with to become pathogenic are also present. Some of these factors are unknown, but the observation that some patients with high anti-dsDNA antibody levels relapsed only when B cells were present suggests that anti-dsDNA antibodies are dependent on them either directly or indirectly. Some biomarkers might only be useful at specific phases of SLE. For example, circulating numbers of $CD4^{+}CD49d^{hi}HLA\text{-}DR^{+}$ T cells might be useful at an intermediate phase in patients with Type A disease, before ELT has formed and anti-dsDNA antibodies are produced.

This thesis provides a possible method for stratifying patients by grouping them according to differences in their T cell phenotypes. These groups appear to be able to predict whether anti-dsDNA antibody levels will change after therapy, i.e. according to which inflammatory pathway is dominant, when their B cells will repopulate, if and when T cell numbers will fall and they might also predict the time to clinical relapse. Further work is needed to confirm these findings and also examine whether these different groups show differences in the time

to clinical response. If they do show differences in the time to clinical response and/or relapse then the time for assessing the primary end-points for each group will need to be adjusted accordingly.

In the introduction it was suggested that the immune response should be considered as distinct phases; lymphopoieisis (for the primary response) or formation of the memory pool (for the secondary response), then surveillance, antigen recognition, clonal expansion and differentiation (activation), antigen elimination and finally contraction. Most studies have suggested that SLE is caused by inappropriate antigen recognition (562), excessive activation (563) and/or failure of the immune response to contract (564). In this thesis I have described T cells that are terminally differentiated with central and effector memory phenotypes, suggesting that there is a large memory pool, which is not affected by BCDT. The effector memory T cells are primed to survey the peripheral non-lymphoid tissue where they instruct B cells to become plasma cells that make large numbers autoantibodies inside the tissue. The central memory T cells prime B cells within SLOs to form plasma cells that make circulating autoantibodies that survey the blood and tissue when the endothelium allows antibodies to cross over from the circulation. I therefore believe that SLE should instead be seen as a disease of inappropriate surveillance of non-lymphoid tissue by primed memory T cells (Type A) and antibodies (Type B). It is likely that most therapies achieve remission (or disease maintenance) by inhibiting one or both of these two processes.

Most patients relapse at some point after BCDT, which shows that "disease memory" is not eliminated by the removal of B cells, confirmed by the fact that memory T cell phenotypes do not change. A disease cure might be possible if these memory T cells can either be depleted or reverted to naïve. Such a therapy might carry a high risk of infection but anti-IL-6 therapy has shown that reverting T cells to a naïve phenotype can be achieved (471) and this should possibly be the main focus for future research.

This thesis provides a new understanding of the pathogenetic sequences that lead to clinical disease, a potentially novel method for stratifying patients and biomarkers to quantify disease activity. Future work should attempt to validate this model, both in animal models and in prospective human studies, the biomarkers that have been identified for each phase, and understand the mechanisms of each phase. In Type A disease there are the following questions for each phase; Phase 1 – what are the genetic and environmental factors that predispose to disease? Phase 2 – what factors mediate T and B cell differentiation and what is the precursor of the $IgD^{-}CD27^{-}$ B cell? Phase 3 & 4 – does HLA-DR effect T cell migration and what is the mechanism? Phase 5 – what factors recruit B cells to join T cell aggregates in the non-lymphoid tissue and where do they originate? Phase 6 – are FDCs necessary for the formation of anti-dsDNA antibody producing plasma cells? Phase 7 – are anti-dsDNA antibodies from ELT the same as anti-dsDNA antibodies from SLOs or are there functional differences? Phase 8 – what role do anti-dsDNA antibodies have? Do T cells follow anti-dsDNA antibodies and do they cause endothelial dyregulation? Phase 9 – how does the adhesion molecule VLA-4 contribute to vasculitis? Do anti-dsDNA antibodies also have a role in vasculitis? Phase 10 – are APL antibodies produced in SLOs by LLPCs or in ELT by SLPCs? In Type B disease there are the following questions; Phase 1 – what are the genetic and environmental factors that predispose to disease? Phase 2 – what factors mediate T and B cell differentiation? Phase 3 – what factors lead to the survival of LLPCs. The sequence of events after LLPCs are produced then need establishing. As mentioned previously, one particular question is what are the mechanisms that allow antibodies to cross the endothelium and how do they then induce damage? In mixed disease, does the combination of the two different disease pathways lead to symptoms and pathology that are a mixture of the other two types of disease or a different group of symptoms?

Summary

In order to develop effective therapies, we need to be able to develop markers that can predict the outcome of the disease (prognostic markers) and quantify the progression of the disease. These markers will be found when there is a better understanding of the disease pathogenesis. Most studies have focused on the autoantibodies and the regulatory processes that lead to the production of these antibodies, but this thesis has instead tried to use B cell depletion to examine where pathological anti-dsDNA antibodies are produced and how these antibodies might then cross the endothelium to get into the tissue. It has identified different patterns of T cell migration and a possible unique mechanism of T cell activation by B cells that induces T cell migration to the non-lymphoid tissue. In doing so, this thesis describes two distinct processes, which can be predicted and quantified. It will hopefully be possible to use these markers for future clinical trials.

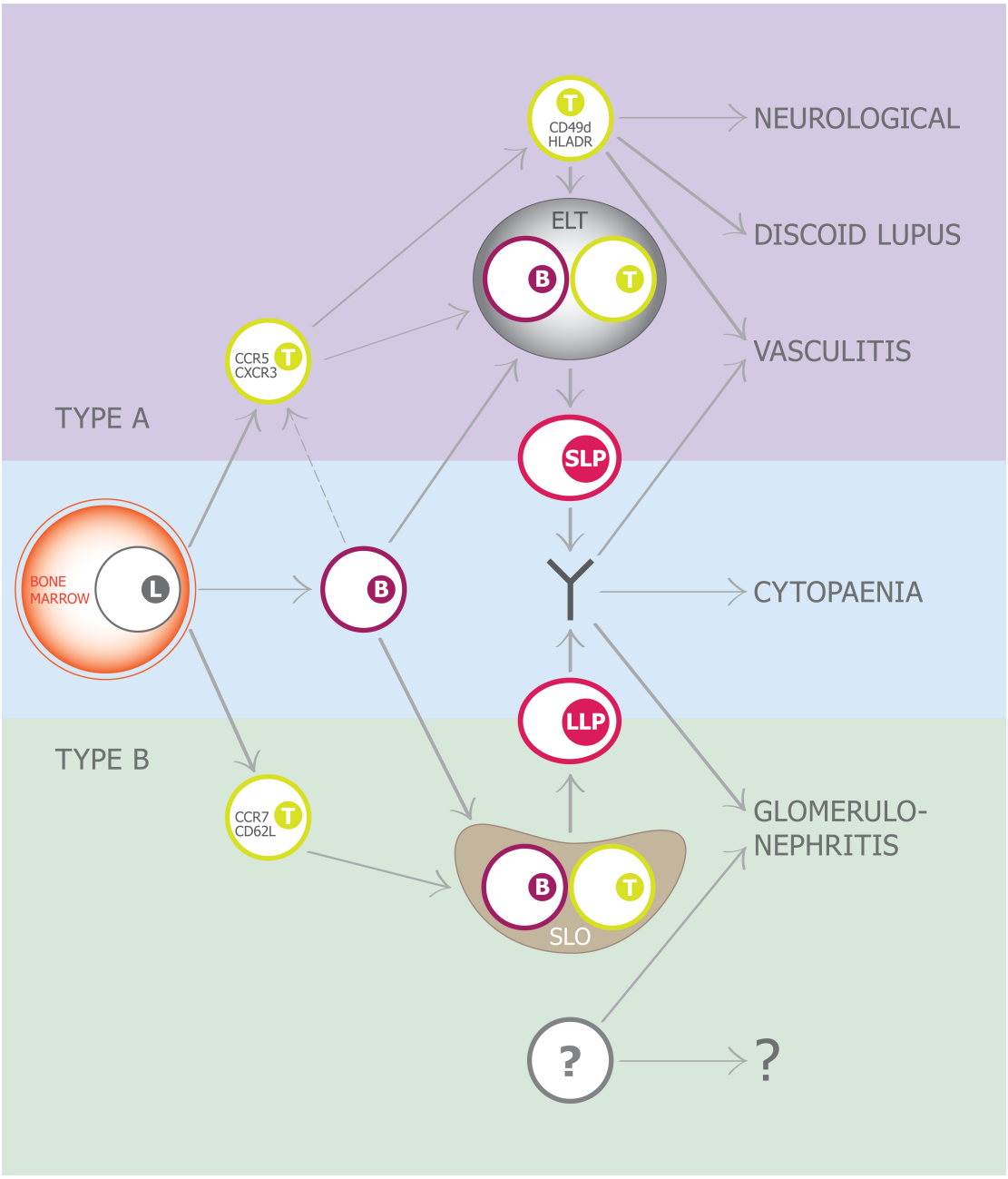


Figure 6.1.1 – A proposed model of SLE

This thesis proposes that B and T cells have multiple roles in SLE, which result in different symptoms and outcomes from therapy, in particular BCDT. It also proposes that the disease can be divided into two broad groups, based on where B and T cells interact. Type A disease is characterized by B-T cell interactions in ectopic lymphoid tissue (ELT), which result in short-lived plasma cells (SLP). Patients with type A disease can be identified by $CD4^+$ T cells that express CCR5, CXCR3 and CD49d. Type A disease manifestations are likely to initially be due to interstitial infiltrates (e.g. discoid lupus) followed by autoantibody production (e.g. vasculitis). Type B disease is characterized by B-T cell interactions in secondary lymphoid organs (SLO), which result in long-lived plasma cells (LLP). Patients with type B disease can be identified by $CD4^+$ T cells that express CCR7 and CD62L. Type B disease manifestations are likely to be due to immune complex deposition combined with other factors, such as neutrophils (e.g. glomerulonephritis). Some symptoms might arise via either one of these two proposed pathways (e.g. cytopaenia) or as a result of a combination of these two pathways.

	Type A	Type B
Circulating T cells	**Effector/Revertant** **High CD49d, CCR5 and CXCR3** Low CCR7 and CD62L	**Naïve/Central** Low CD49d, CCR5 and CXCR3 **High CCR7 and CD62L**
T cell activation marker	**HLA-DR**^{hi} (on CD49d^{hi} T cells)	CD69?
Circulating B cells	**Double Negative**	**Plasmablasts**
DNA Ab producing Plasma cells	**Short-lived** DNA Abs return to normal after BCDT	**Long-lived** DNA Abs remain high after BCDT
Chemokines	-	**High MCP-1 and IP-10**
Relationship between circulating leukocytes and DNA Abs	**Lymphocytes (T cells) fall before rise in DNA Abs and clinical relapse**	**DNA Abs are persistently high.** Lymphocytes and monocytes fall during clinical relapse
Source of DNA Ab production	**Ectopic Lymphoid Tissue**	Secondary Lymphoid Organs
Role of DNA Abs	Endothelial activation?	Subendothelial immune complex deposition
Clinical disease	Localized disease (ELT formation) with cerebral lesions, arthritis or focal rash followed by vasculitis. Chronic, progressive disease.	Systemic disease with constitutional symptoms, diffuse rash and glomerulonephritis Acute, intermittent disease.
Pathology	Schwartzman reaction (Type 4 Hypersensitivity?) Endothelial damage	Arthus reaction (Type 3 Hypersensitivity) Epithelial damage?
Response to BCDT	Fast clinical response but relapse shortly after B cells return	Slow clinical response but B cell return is not associated with relapse

Table 6.1.1 – Summary of proposed SLE subtypes.
Clinical, serological and cellular differences that have been observed and described in this thesis are written in bold letters. Possible differences that require further investigation are written in normal letters.

References

1. Abbas AK, Lichtman AH, Pillai S. 2010. *Cellular and molecular immunology*. Philadelphia: Saunders/Elsevier. viii, 566 p. pp.
2. Davenport MP, Belz GT, Ribeiro RM. 2009. The race between infection and immunity: how do pathogens set the pace? *Trends Immunol* 30: 61-6
3. Johnson S. 1997. Antibody responses to clostridial infection in humans. *Clin Infect Dis* 25 Suppl 2: S173-7
4. Randow F, MacMicking JD, James LC. 2013. Cellular self-defense: how cell-autonomous immunity protects against pathogens. *Science* 340: 701-6
5. Tsiantoulas D, Gruber S, Binder CJ. 2012. B-1 cell immunoglobulin directed against oxidation-specific epitopes. *Front Immunol* 3: 415
6. Cinti S, Mitchell G, Barbatelli G, Murano I, Ceresi E, Faloia E, Wang S, Fortier M, Greenberg AS, Obin MS. 2005. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res* 46: 2347-55
7. Peng Y, Kowalewski R, Kim S, Elkon KB. 2005. The role of IgM antibodies in the recognition and clearance of apoptotic cells. *Mol Immunol* 42: 781-7
8. Ravichandran KS. 2010. Find-me and eat-me signals in apoptotic cell clearance: progress and conundrums. *J Exp Med* 207: 1807-17
9. Finn OJ. 2008. Cancer immunology. *N Engl J Med* 358: 2704-15
10. Braff MH, Bardan A, Nizet V, Gallo RL. 2005. Cutaneous defense mechanisms by antimicrobial peptides. *J Invest Dermatol* 125: 9-13
11. Pearlman E, Sun Y, Roy S, Karmakar M, Hise AG, Szczotka-Flynn L, Ghannoum M, Chinnery HR, McMenamin PG, Rietsch A. 2013. Host defense at the ocular surface. *Int Rev Immunol* 32: 4-18
12. Wiedle G, Dunon D, Imhof BA. 2001. Current concepts in lymphocyte homing and recirculation. *Crit Rev Clin Lab Sci* 38: 1-31
13. Gao B, Jeong WI, Tian Z. 2008. Liver: An organ with predominant innate immunity. *Hepatology* 47: 729-36
14. Jenne CN, Kubes P. 2013. Immune surveillance by the liver. *Nat Immunol* 14: 996-1006
15. Pearson C, Uhlig HH, Powrie F. 2012. Lymphoid microenvironments and innate lymphoid cells in the gut. *Trends Immunol* 33: 289-96
16. Ransohoff RM, Brown MA. 2012. Innate immunity in the central nervous system. *J Clin Invest* 122: 1164-71
17. Gordon S. 2002. Pattern recognition receptors: doubling up for the innate immune response. *Cell* 111: 927-30
18. Takeuchi O, Akira S. 2010. Pattern recognition receptors and inflammation. *Cell* 140: 805-20
19. Beutler B. 2004. Innate immunity: an overview. *Mol Immunol* 40: 845-59
20. Crampton SP, Voynova E, Bolland S. 2010. Innate pathways to B-cell activation and tolerance. *Ann N Y Acad Sci* 1183: 58-68
21. Reynolds JM, Pappu BP, Peng J, Martinez GJ, Zhang Y, Chung Y, Ma L, Yang XO, Nurieva RI, Tian Q, Dong C. 2010. Toll-like receptor 2 signaling in CD4(+) T lymphocytes promotes T helper 17 responses and regulates the pathogenesis of autoimmune disease. *Immunity* 32: 692-702
22. Boehm T. 2012. Evolution of vertebrate immunity. *Curr Biol* 22: R722-32
23. MacLeod MK, Kappler JW, Marrack P. 2010. Memory CD4 T cells: generation, reactivation and re-assignment. *Immunology* 130: 10-5
24. Kalia V, Sarkar S, Gourley TS, Rouse BT, Ahmed R. 2006. Differentiation of memory B and T cells. *Curr Opin Immunol* 18: 255-64
25. Campos M, Godson DL. 2003. The effectiveness and limitations of immune memory: understanding protective immune responses. *Int J Parasitol* 33: 655-61

26. Orteu CH, Poulter LW, Rustin MH, Sabin CA, Salmon M, Akbar AN. 1998. The role of apoptosis in the resolution of T cell-mediated cutaneous inflammation. *J Immunol* 161: 1619-29
27. Bonilla FA, Oettgen HC. 2010. Adaptive immunity. *J Allergy Clin Immunol* 125: S33-40
28. Metzger TC, Anderson MS. 2011. Control of central and peripheral tolerance by Aire. *Immunol Rev* 241: 89-103
29. Yarkoni Y, Getahun A, Cambier JC. 2010. Molecular underpinning of B-cell anergy. *Immunol Rev* 237: 249-63
30. Akashi K, Reya T, Dalma-Weiszhausz D, Weissman IL. 2000. Lymphoid precursors. *Curr Opin Immunol* 12: 144-50
31. Allman D, Pillai S. 2008. Peripheral B cell subsets. *Curr Opin Immunol* 20: 149-57
32. Busslinger M. 2004. Transcriptional control of early B cell development. *Annu Rev Immunol* 22: 55-79
33. Anolik JH, Barnard J, Cappione A, Pugh-Bernard AE, Felgar RE, Looney RJ, Sanz I. 2004. Rituximab improves peripheral B cell abnormalities in human systemic lupus erythematosus. *Arthritis Rheum* 50: 3580-90
34. Casola S. 2007. Control of peripheral B-cell development. *Curr Opin Immunol* 19: 143-9
35. Vinuesa CG, Linterman MA, Goodnow CC, Randall KL. 2010. T cells and follicular dendritic cells in germinal center B-cell formation and selection. *Immunol Rev* 237: 72-89
36. MacLennan IC, Toellner KM, Cunningham AF, Serre K, Sze DM, Zuniga E, Cook MC, Vinuesa CG. 2003. Extrafollicular antibody responses. *Immunol Rev* 194: 8-18
37. Pereira JP, Kelly LM, Cyster JG. 2010. Finding the right niche: B-cell migration in the early phases of T-dependent antibody responses. *Int Immunol* 22: 413-9
38. Vinuesa CG, Chang PP. 2013. Innate B cell helpers reveal novel types of antibody responses. *Nat Immunol* 14: 119-26
39. Onah DN, Nawa Y. 2004. Mucosal mast cell-derived chondroitin sulphate levels in and worm expulsion from FcRgamma-knockout mice following oral challenge with Strongyloides venezuelensis. *J Vet Sci* 5: 221-6
40. Gurish MF, Bryce PJ, Tao H, Kisselgof AB, Thornton EM, Miller HR, Friend DS, Oettgen HC. 2004. IgE enhances parasite clearance and regulates mast cell responses in mice infected with Trichinella spiralis. *J Immunol* 172: 1139-45
41. Vidarsson G, Dekkers G, Rispens T. 2014. IgG subclasses and allotypes: from structure to effector functions. *Front Immunol* 5: 520
42. Notarangelo LD, Lanzi G, Peron S, Durandy A. 2006. Defects of class-switch recombination. *J Allergy Clin Immunol* 117: 855-64
43. Stavnezer J, Guikema JE, Schrader CE. 2008. Mechanism and regulation of class switch recombination. *Annu Rev Immunol* 26: 261-92
44. Kurt-Jones EA, Liano D, HayGlass KA, Benacerraf B, Sy MS, Abbas AK. 1988. The role of antigen-presenting B cells in T cell priming in vivo. Studies of B cell-deficient mice. *J Immunol* 140: 3773-8
45. Crawford A, Macleod M, Schumacher T, Corlett L, Gray D. 2006. Primary T cell expansion and differentiation in vivo requires antigen presentation by B cells. *J Immunol* 176: 3498-506
46. Whitmire JK, Asano MS, Kaech SM, Sarkar S, Hannum LG, Shlomchik MJ, Ahmed R. 2009. Requirement of B cells for generating CD4+ T cell memory. *J Immunol* 182: 1868-76
47. Gray D, Gray M, Barr T. 2007. Innate responses of B cells. *Eur J Immunol* 37: 3304-10
48. Nakayama T, Hieshima K, Nagakubo D, Sato E, Nakayama M, Kawa K, Yoshie O. 2004. Selective induction of Th2-attracting chemokines CCL17 and CCL22 in human B cells by latent membrane protein 1 of Epstein-Barr virus. *J Virol* 78: 1665-74

49. Vollmer J, Jurk M, Samulowitz U, Lipford G, Forsbach A, Wullner M, Tluk S, Hartmann H, Kritzler A, Muller C, Schetter C, Krieg AM. 2004. CpG oligodeoxynucleotides stimulate IFN-gamma-inducible protein-10 production in human B cells. *J Endotoxin Res* 10: 431-8
50. Kato A, Ogasawara T, Homma T, Batchelor J, Imai S, Wakiguchi H, Saito H, Matsumoto K. 2004. CpG oligodeoxynucleotides directly induce CXCR3 chemokines in human B cells. *Biochem Biophys Res Commun* 320: 1139-47
51. Linton PJ, Bautista B, Biederman E, Bradley ES, Harbertson J, Kondrack RM, Padrick RC, Bradley LM. 2003. Costimulation via OX40L expressed by B cells is sufficient to determine the extent of primary CD4 cell expansion and Th2 cytokine secretion in vivo. *J Exp Med* 197: 875-83
52. Barr TA, Brown S, Mastroeni P, Gray D. 2010. TLR and B cell receptor signals to B cells differentially program primary and memory Th1 responses to Salmonella enterica. *J Immunol* 185: 2783-9
53. Wojciechowski W, Harris DP, Sprague F, Mousseau B, Makris M, Kusser K, Honjo T, Mohrs K, Mohrs M, Randall T, Lund FE. 2009. Cytokine-producing effector B cells regulate type 2 immunity to H. polygyrus. *Immunity* 30: 421-33
54. Klein J, Sato A. 2000. The HLA system. First of two parts. *N Engl J Med* 343: 702-9
55. Woodland DL, Dutton RW. 2003. Heterogeneity of CD4(+) and CD8(+) T cells. *Curr Opin Immunol* 15: 336-42
56. Annunziato F, Romagnani S. 2009. Heterogeneity of human effector CD4+ T cells. *Arthritis Res Ther* 11: 257
57. Berke G. 1994. The binding and lysis of target cells by cytotoxic lymphocytes: molecular and cellular aspects. *Annu Rev Immunol* 12: 735-73
58. Shevach EM. 2011. Biological functions of regulatory T cells. *Adv Immunol* 112: 137-76
59. Appay V, van Lier RA, Sallusto F, Roederer M. 2008. Phenotype and function of human T lymphocyte subsets: consensus and issues. *Cytometry A* 73: 975-83
60. Zhu J, Paul WE. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112: 1557-69
61. Ma CS, Deenick EK, Batten M, Tangye SG. 2012. The origins, function, and regulation of T follicular helper cells. *J Exp Med* 209: 1241-53
62. Chu VT, Berek C. 2013. The establishment of the plasma cell survival niche in the bone marrow. *Immunol Rev* 251: 177-88
63. Brusko TM, Putnam AL, Bluestone JA. 2008. Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev* 223: 371-90
64. Iezzi G, Scotet E, Scheidegger D, Lanzavecchia A. 1999. The interplay between the duration of TCR and cytokine signaling determines T cell polarization. *Eur J Immunol* 29: 4092-101
65. Deng J, Dekruyff RH, Freeman GJ, Umetsu DT, Levy S. 2002. Critical role of CD81 in cognate T-B cell interactions leading to Th2 responses. *Int Immunol* 14: 513-23
66. Sallusto F, Lanzavecchia A. 2002. The instructive role of dendritic cells on T-cell responses. *Arthritis Res* 4 Suppl 3: S127-32
67. Harris DP, Haynes L, Sayles PC, Duso DK, Eaton SM, Lepak NM, Johnson LL, Swain SL, Lund FE. 2000. Reciprocal regulation of polarized cytokine production by effector B and T cells. *Nat Immunol* 1: 475-82
68. Stevens SK, Weissman IL, Butcher EC. 1982. Differences in the migration of B and T lymphocytes: organ-selective localization in vivo and the role of lymphocyte-endothelial cell recognition. *J Immunol* 128: 844-51
69. Tang ML, Steeber DA, Zhang XQ, Tedder TF. 1998. Intrinsic differences in L-selectin expression levels affect T and B lymphocyte subset-specific recirculation pathways. *J Immunol* 160: 5113-21
70. Gebhardt T, Whitney PG, Zaid A, Mackay LK, Brooks AG, Heath WR, Carbone FR, Mueller SN. 2011. Different patterns of peripheral migration by memory CD4+ and CD8+ T cells. *Nature* 477: 216-9

71. Jiang X, Clark RA, Liu L, Wagers AJ, Fuhlbrigge RC, Kupper TS. 2012. Skin infection generates non-migratory memory CD8+ T(RM) cells providing global skin immunity. *Nature* 483: 227-31
72. Carragher DM, Rangel-Moreno J, Randall TD. 2008. Ectopic lymphoid tissues and local immunity. *Semin Immunol* 20: 26-42
73. Inman CF, Murray TZ, Bailey M, Cose S. 2012. Most B cells in non-lymphoid tissues are naive. *Immunol Cell Biol* 90: 235-42
74. Bleul CC, Schultze JL, Springer TA. 1998. B lymphocyte chemotaxis regulated in association with microanatomic localization, differentiation state, and B cell receptor engagement. *J Exp Med* 187: 753-62
75. Kunkel EJ, Butcher EC. 2003. Plasma-cell homing. *Nat Rev Immunol* 3: 822-9
76. Mamani-Matsuda M, Cosma A, Weller S, Faili A, Staib C, Garcon L, Hermine O, Beyne-Rauzy O, Fieschi C, Pers JO, Arakelyan N, Varet B, Sauvanet A, Berger A, Paye F, Andrieu JM, Michel M, Godeau B, Buffet P, Reynaud CA, Weill JC. 2008. The human spleen is a major reservoir for long-lived vaccinia virus-specific memory B cells. *Blood* 111: 4653-9
77. Rupprecht TA, Plate A, Adam M, Wick M, Kastenbauer S, Schmidt C, Klein M, Pfister HW, Koedel U. 2009. The chemokine CXCL13 is a key regulator of B cell recruitment to the cerebrospinal fluid in acute Lyme neuroborreliosis. *J Neuroinflammation* 6: 42
78. Narayan K, Dail D, Li L, Cadavid D, Amrute S, Fitzgerald-Bocarsly P, Pachner AR. 2005. The nervous system as ectopic germinal center: CXCL13 and IgG in lyme neuroborreliosis. *Ann Neurol* 57: 813-23
79. Xu B, Wagner N, Pham LN, Magno V, Shan Z, Butcher EC, Michie SA. 2003. Lymphocyte homing to bronchus-associated lymphoid tissue (BALT) is mediated by L-selectin/PNAd, alpha4beta1 integrin/VCAM-1, and LFA-1 adhesion pathways. *J Exp Med* 197: 1255-67
80. Engelhardt B. 2006. Molecular mechanisms involved in T cell migration across the blood-brain barrier. *J Neural Transm* 113: 477-85
81. Man S, Tucky B, Cotleur A, Drazba J, Takeshita Y, Ransohoff RM. 2012. CXCL12-induced monocyte-endothelial interactions promote lymphocyte transmigration across an in vitro blood-brain barrier. *Sci Transl Med* 4: 119ra14
82. Ludowyk PA, Willenborg DO, Parish CR. 1992. Selective localisation of neuro-specific T lymphocytes in the central nervous system. *J Neuroimmunol* 37: 237-50
83. Archambault AS, Sim J, Gimenez MA, Russell JH. 2005. Defining antigen-dependent stages of T cell migration from the blood to the central nervous system parenchyma. *Eur J Immunol* 35: 1076-85
84. Galea I, Bernardes-Silva M, Forse PA, van Rooijen N, Liblau RS, Perry VH. 2007. An antigen-specific pathway for CD8 T cells across the blood-brain barrier. *J Exp Med* 204: 2023-30
85. Knopf PM, Harling-Berg CJ, Cserr HF, Basu D, Sirulnick EJ, Nolan SC, Park JT, Keir G, Thompson EJ, Hickey WF. 1998. Antigen-dependent intrathecal antibody synthesis in the normal rat brain: tissue entry and local retention of antigen-specific B cells. *J Immunol* 161: 692-701
86. Hooper DC, Phares TW, Fabis MJ, Roy A. 2009. The production of antibody by invading B cells is required for the clearance of rabies virus from the central nervous system. *PLoS Negl Trop Dis* 3: e535
87. Alter A, Duddy M, Hebert S, Biernacki K, Prat A, Antel JP, Yong VW, Nuttall RK, Pennington CJ, Edwards DR, Bar-Or A. 2003. Determinants of human B cell migration across brain endothelial cells. *J Immunol* 170: 4497-505
88. Marelli-Berg FM, Fu H, Vianello F, Tokoyoda K, Hamann A. 2010. Memory T-cell trafficking: new directions for busy commuters. *Immunology* 130: 158-65
89. Thomas W, Cunningham P. 2009. Hypersensitivity: Immunological. In *eLS. John Wiley & Sons Ltd, Chichester*

90. Goyette P, Labbe C, Trinh TT, Xavier RJ, Rioux JD. 2007. Molecular pathogenesis of inflammatory bowel disease: genotypes, phenotypes and personalized medicine. *Ann Med* 39: 177-99
91. McGonagle D, McDermott MF. 2006. A proposed classification of the immunological diseases. *PLoS Med* 3: e297
92. Toubi E, Shoenfeld Y. 2007. Protective autoimmunity in cancer (review). *Oncol Rep* 17: 245-51
93. Kyaw T, Tipping P, Bobik A, Toh BH. 2012. Protective role of natural IgM-producing B1a cells in atherosclerosis. *Trends Cardiovasc Med* 22: 48-53
94. Mannoor K, Li C, Inafuku M, Taniguchi T, Abo T, Sato Y, Watanabe H. 2013. Induction of ssDNA-binding autoantibody secreting B cell immunity during murine malaria infection is a critical part of the protective immune responses. *Immunobiology* 218: 10-20
95. Moalem G, Leibowitz-Amit R, Yoles E, Mor F, Cohen IR, Schwartz M. 1999. Autoimmune T cells protect neurons from secondary degeneration after central nervous system axotomy. *Nat Med* 5: 49-55
96. Goyal R, Bulua AC, Nikolov NP, Schwartzberg PL, Siegel RM. 2009. Rheumatologic and autoimmune manifestations of primary immunodeficiency disorders. *Curr Opin Rheumatol* 21: 78-84
97. Zandman-Goddard G, Shoenfeld Y. 2002. HIV and autoimmunity. *Autoimmun Rev* 1: 329-37
98. Rahman A, Isenberg DA. 2008. Systemic lupus erythematosus. *N Engl J Med* 358: 929-39
99. Dhillon PK, Adams MJ. 2013. Thrombosis in systemic lupus erythematosus: role of impaired fibrinolysis. *Semin Thromb Hemost* 39: 434-40
100. Schoenfeld SR, Kasturi S, Costenbader KH. 2013. The epidemiology of atherosclerotic cardiovascular disease among patients with SLE: a systematic review. *Semin Arthritis Rheum* 43: 77-95
101. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, Schaller JG, Talal N, Winchester RJ. 1982. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 25: 1271-7
102. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, Bruce IN, Isenberg D, Wallace DJ, Nived O, Sturfelt G, Ramsey-Goldman R, Bae SC, Hanly JG, Sanchez-Guerrero J, Clarke A, Aranow C, Manzi S, Urowitz M, Gladman D, Kalunian K, Costner M, Werth VP, Zoma A, Bernatsky S, Ruiz-Irastorza G, Khamashta MA, Jacobsen S, Buyon JP, Maddison P, Dooley MA, van Vollenhoven RF, Ginzler E, Stoll T, Peschken C, Jorizzo JL, Callen JP, Lim SS, Fessler BJ, Inanc M, Kamen DL, Rahman A, Steinsson K, Franks AG, Jr., Sigler L, Hameed S, Fang H, Pham N, Brey R, Weisman MH, McGwin G, Jr., Magder LS. 2012. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* 64: 2677-86
103. D'Cruz D. 2002. Testing for autoimmunity in humans. *Toxicol Lett* 127: 93-100
104. Maroz N, Segal MS. 2013. Lupus nephritis and end-stage kidney disease. *Am J Med Sci* 346: 319-23
105. Jacobsen S, Petersen J, Ullman S, Junker P, Voss A, Rasmussen JM, Tarp U, Poulsen LH, van Overeem Hansen G, Skaarup B, Hansen TM, Podenphant J, Halberg P. 1998. A multicentre study of 513 Danish patients with systemic lupus erythematosus. I. Disease manifestations and analyses of clinical subsets. *Clin Rheumatol* 17: 468-77
106. To CH, Mok CC, Tang SS, Ying SK, Wong RW, Lau CS. 2009. Prognostically distinct clinical patterns of systemic lupus erythematosus identified by cluster analysis. *Lupus* 18: 1267-75
107. Arbuckle MR, McClain MT, Rubertone MV, Scofield RH, Dennis GJ, James JA, Harley JB. 2003. Development of autoantibodies before the clinical onset of systemic lupus erythematosus. *N Engl J Med* 349: 1526-33

108. Greer JM, Panush RS. 1989. Incomplete lupus erythematosus. *Arch Intern Med* 149: 2473-6
109. Ganczarczyk L, Urowitz MB, Gladman DD. 1989. "Latent lupus". *J Rheumatol* 16: 475-8
110. Vila LM, Mayor AM, Valentin AH, Garcia-Soberal M, Vila S. 2000. Clinical outcome and predictors of disease evolution in patients with incomplete lupus erythematosus. *Lupus* 9: 110-5
111. Swaak AJ, van de Brink H, Smeenk RJ, Manger K, Kalden JR, Tosi S, Marchesoni A, Domljan Z, Rozman B, Logar D, Pokorny G, Kovacs L, Kovacs A, Vlachoyiannopoulos PG, Moutsopoulos HM, Chwalinska-Sadowska H, Dratwianka B, Kiss E, Cikes N, Anic B, Schneider M, Fischer R, Bombardieri S, Mosca M, Graninger W, Smolen JS, Study group on incomplete SLE, years SLEwddlt. 2001. Incomplete lupus erythematosus: results of a multicentre study under the supervision of the EULAR Standing Committee on International Clinical Studies Including Therapeutic Trials (ESCISIT). *Rheumatology (Oxford)* 40: 89-94
112. Stahl Hallengren C, Nived O, Sturfelt G. 2004. Outcome of incomplete systemic lupus erythematosus after 10 years. *Lupus* 13: 85-8
113. Laustrup H, Voss A, Green A, Junker P. 2009. Occurrence of systemic lupus erythematosus in a Danish community: an 8-year prospective study. *Scand J Rheumatol* 38: 128-32
114. Vaughn SE, Kottyan LC, Munroe ME, Harley JB. 2012. Genetic susceptibility to lupus: the biological basis of genetic risk found in B cell signaling pathways. *J Leukoc Biol* 92: 577-91
115. Glass D, Raum D, Gibson D, Stillman JS, Schur PH. 1976. Inherited deficiency of the second component of complement. Rheumatic disease associations. *J Clin Invest* 58: 853-61
116. Rhodes B, Vyse TJ. 2008. The genetics of SLE: an update in the light of genome-wide association studies. *Rheumatology (Oxford)* 47: 1603-11
117. Deng Y, Tsao BP. 2010. Genetic susceptibility to systemic lupus erythematosus in the genomic era. *Nat Rev Rheumatol* 6: 683-92
118. Weckerle CE, Niewold TB. 2011. The unexplained female predominance of systemic lupus erythematosus: clues from genetic and cytokine studies. *Clin Rev Allergy Immunol* 40: 42-9
119. Petri M. 2008. Sex hormones and systemic lupus erythematosus. *Lupus* 17: 412-5
120. Sebastiani GD, Galeazzi M. 2009. Infection--genetics relationship in systemic lupus erythematosus. *Lupus* 18: 1169-75
121. Cooper GS, Parks CG. 2004. Occupational and environmental exposures as risk factors for systemic lupus erythematosus. *Curr Rheumatol Rep* 6: 367-74
122. Kuhn A, Beissert S. 2005. Photosensitivity in lupus erythematosus. *Autoimmunity* 38: 519-29
123. Chang C, Gershwin ME. 2010. Drugs and autoimmunity--a contemporary review and mechanistic approach. *J Autoimmun* 34: J266-75
124. Romero-Diaz J, Isenberg D, Ramsey-Goldman R. 2011. Measures of adult systemic lupus erythematosus: updated version of British Isles Lupus Assessment Group (BILAG 2004), European Consensus Lupus Activity Measurements (ECLAM), Systemic Lupus Activity Measure, Revised (SLAM-R), Systemic Lupus Activity Questionnaire for Population Studies (SLAQ), Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K), and Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index (SDI). *Arthritis Care Res (Hoboken)* 63 Suppl 11: S37-46
125. Mittal B, Rennke H, Singh AK. 2005. The role of kidney biopsy in the management of lupus nephritis. *Curr Opin Nephrol Hypertens* 14: 1-8
126. Baltaci M, Fritsch P. 2009. Histologic features of cutaneous lupus erythematosus. *Autoimmun Rev* 8: 467-73

127. Reveille JD. 2004. Predictive value of autoantibodies for activity of systemic lupus erythematosus. *Lupus* 13: 290-7
128. Jacobi AM, Odendahl M, Reiter K, Bruns A, Burmester GR, Radbruch A, Valet G, Lipsky PE, Dorner T. 2003. Correlation between circulating CD27high plasma cells and disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* 48: 1332-42
129. Kohro-Kawata J, Wener MH, Mannik M. 2002. The effect of high salt concentration on detection of serum immune complexes and autoantibodies to C1q in patients with systemic lupus erythematosus. *J Rheumatol* 29: 84-9
130. Illei GG, Tackey E, Lapteva L, Lipsky PE. 2004. Biomarkers in systemic lupus erythematosus: II. Markers of disease activity. *Arthritis Rheum* 50: 2048-65
131. Sturfelt G, Truedsson L. 2005. Complement and its breakdown products in SLE. *Rheumatology (Oxford)* 44: 1227-32
132. Gladman DD, Hirani N, Ibanez D, Urowitz MB. 2003. Clinically active serologically quiescent systemic lupus erythematosus. *J Rheumatol* 30: 1960-2
133. Reyes-Thomas J, Blanco I, Putterman C. 2011. Urinary biomarkers in lupus nephritis. *Clin Rev Allergy Immunol* 40: 138-50
134. Enghard P, Humrich JY, Rudolph B, Rosenberger S, Biesen R, Kuhn A, Manz R, Hiepe F, Radbruch A, Burmester GR, Riemekasten G. 2009. CXCR3+CD4+ T cells are enriched in inflamed kidneys and urine and provide a new biomarker for acute nephritis flares in systemic lupus erythematosus patients. *Arthritis Rheum* 60: 199-206
135. Duval A, Helley D, Capron L, Youinou P, Renaudineau Y, Dubucquoi S, Fischer AM, Hachulla E. 2010. Endothelial dysfunction in systemic lupus patients with low disease activity: evaluation by quantification and characterization of circulating endothelial microparticles, role of anti-endothelial cell antibodies. *Rheumatology (Oxford)* 49: 1049-55
136. Boulanger CM, Amabile N, Tedgui A. 2006. Circulating microparticles: a potential prognostic marker for atherosclerotic vascular disease. *Hypertension* 48: 180-6
137. Liu CC, Ahearn JM. 2009. The search for lupus biomarkers. *Best Pract Res Clin Rheumatol* 23: 507-23
138. Harris EN, Gharavi AE, Boey ML, Patel BM, Mackworth-Young CG, Loizou S, Hughes GR. 1983. Anticardiolipin antibodies: detection by radioimmunoassay and association with thrombosis in systemic lupus erythematosus. *Lancet* 2: 1211-4
139. Deleze M, Alarcon-Segovia D, Oria CV, Sanchez-Guerrero J, Fernandez-Dominguez L, Gomez-Pacheco L, Ponce de Leon S. 1989. Hemocytopenia in systemic lupus erythematosus. Relationship to antiphospholipid antibodies. *J Rheumatol* 16: 926-30
140. Hoffman IE, Peene I, Meheus L, Huizinga TW, Cebecauer L, Isenberg D, De Bosschere K, Hulstaert F, Veys EM, De Keyser F. 2004. Specific antinuclear antibodies are associated with clinical features in systemic lupus erythematosus. *Ann Rheum Dis* 63: 1155-8
141. Ioannou Y, Isenberg DA. 2002. Current concepts for the management of systemic lupus erythematosus in adults: a therapeutic challenge. *Postgrad Med J* 78: 599-606
142. Kandala NB, Connock M, Grove A, Sutcliffe P, Mohiuddin S, Hartley L, Court R, Cummins E, Gordon C, Clarke A. 2013. Belimumab: a technological advance for systemic lupus erythematosus patients? Report of a systematic review and meta-analysis. *BMJ Open* 3
143. Bertsias G, Boumpas DT. 2008. Update on the management of lupus nephritis: let the treatment fit the patient. *Nat Clin Pract Rheumatol* 4: 464-72
144. Urowitz MB, Gladman DD, Abu-Shakra M, Farewell VT. 1997. Mortality studies in systemic lupus erythematosus. Results from a single center. III. Improved survival over 24 years. *J Rheumatol* 24: 1061-5
145. Alarcon GS, McGwin G, Bertoli AM, Fessler BJ, Calvo-Alen J, Bastian HM, Vila LM, Reveille JD, Group LS. 2007. Effect of hydroxychloroquine on the survival of

patients with systemic lupus erythematosus: data from LUMINA, a multiethnic US cohort (LUMINA L). *Ann Rheum Dis* 66: 1168-72
146. Bruce IN, Gordon C, Merrill JT, Isenberg D. 2010. Clinical trials in lupus: what have we learned so far? *Rheumatology (Oxford)* 49: 1025-7
147. Merrill JT, Burgos-Vargas R, Westhovens R, Chalmers A, D'Cruz D, Wallace DJ, Bae SC, Sigal L, Becker JC, Kelly S, Raghupathi K, Li T, Peng Y, Kinaszczuk M, Nash P. 2010. The efficacy and safety of abatacept in patients with non-life-threatening manifestations of systemic lupus erythematosus: results of a twelve-month, multicenter, exploratory, phase IIb, randomized, double-blind, placebo-controlled trial. *Arthritis Rheum* 62: 3077-87
148. Wallace DJ, Stohl W, Furie RA, Lisse JR, McKay JD, Merrill JT, Petri MA, Ginzler EM, Chatham WW, McCune WJ, Fernandez V, Chevrier MR, Zhong ZJ, Freimuth WW. 2009. A phase II, randomized, double-blind, placebo-controlled, dose-ranging study of belimumab in patients with active systemic lupus erythematosus. *Arthritis Rheum* 61: 1168-78
149. Yildirim-Toruner C, Diamond B. 2011. Current and novel therapeutics in the treatment of systemic lupus erythematosus. *J Allergy Clin Immunol* 127: 303-12; quiz 13-4
150. Murphy G, Lisnevskaia L, Isenberg D. 2013. Systemic lupus erythematosus and other autoimmune rheumatic diseases: challenges to treatment. *Lancet* 382: 809-18
151. Pisetsky DS. 1998. Antibody responses to DNA in normal immunity and aberrant immunity. *Clin Diagn Lab Immunol* 5: 1-6
152. Chang A, Henderson SG, Brandt D, Liu N, Guttikonda R, Hsieh C, Kaverina N, Utset TO, Meehan SM, Quigg RJ, Meffre E, Clark MR. 2011. In situ B cell-mediated immune responses and tubulointerstitial inflammation in human lupus nephritis. *J Immunol* 186: 1849-60
153. Shen Y, Sun CY, Wu FX, Chen Y, Dai M, Yan YC, Yang CD. 2012. Association of intrarenal B-cell infiltrates with clinical outcome in lupus nephritis: a study of 192 cases. *Clin Dev Immunol* 2012: 967584
154. Alexopoulos E, Seron D, Hartley RB, Cameron JS. 1990. Lupus nephritis: correlation of interstitial cells with glomerular function. *Kidney Int* 37: 100-9
155. Couzi L, Merville P, Deminiere C, Moreau JF, Combe C, Pellegrin JL, Viallard JF, Blanco P. 2007. Predominance of CD8+ T lymphocytes among periglomerular infiltrating cells and link to the prognosis of class III and class IV lupus nephritis. *Arthritis Rheum* 56: 2362-70
156. Drenkard C, Villa AR, Reyes E, Abello M, Alarcon-Segovia D. 1997. Vasculitis in systemic lupus erythematosus. *Lupus* 6: 235-42
157. Ramos-Casals M, Nardi N, Lagrutta M, Brito-Zeron P, Bove A, Delgado G, Cervera R, Ingelmo M, Font J. 2006. Vasculitis in systemic lupus erythematosus: prevalence and clinical characteristics in 670 patients. *Medicine (Baltimore)* 85: 95-104
158. Jamin C, Dugue C, Alard JE, Jousse S, Saraux A, Guillevin L, Piette JC, Youinou P. 2005. Induction of endothelial cell apoptosis by the binding of anti-endothelial cell antibodies to Hsp60 in vasculitis-associated systemic autoimmune diseases. *Arthritis Rheum* 52: 4028-38
159. Lai KN, Leung JC, Lai KB, Wong KC, Lai CK. 1996. Upregulation of adhesion molecule expression on endothelial cells by anti-DNA autoantibodies in systemic lupus erythematosus. *Clin Immunol Immunopathol* 81: 229-38
160. Pierangeli SS, Colden-Stanfield M, Liu X, Barker JH, Anderson GL, Harris EN. 1999. Antiphospholipid antibodies from antiphospholipid syndrome patients activate endothelial cells in vitro and in vivo. *Circulation* 99: 1997-2002
161. Carvalho D, Savage CO, Isenberg D, Pearson JD. 1999. IgG anti-endothelial cell autoantibodies from patients with systemic lupus erythematosus or systemic vasculitis stimulate the release of two endothelial cell-derived mediators, which enhance adhesion molecule expression and leukocyte adhesion in an autocrine manner. *Arthritis Rheum* 42: 631-40

162. Belmont HM, Abramson SB, Lie JT. 1996. Pathology and pathogenesis of vascular injury in systemic lupus erythematosus. Interactions of inflammatory cells and activated endothelium. *Arthritis Rheum* 39: 9-22
163. Takeuchi T, Abe T. 2002. 2. Role of adhesion molecules in vasculitis syndrome. *Intern Med* 41: 41-4
164. Takeuchi T, Amano K, Sekine H, Koide J, Abe T. 1993. Upregulated expression and function of integrin adhesive receptors in systemic lupus erythematosus patients with vasculitis. *J Clin Invest* 92: 3008-16
165. Sais G, Vidaller A, Jucgla A, Condom E, Peyri J. 1997. Adhesion molecule expression and endothelial cell activation in cutaneous leukocytoclastic vasculitis. An immunohistologic and clinical study in 42 patients. *Arch Dermatol* 133: 443-50
166. Malide D, Londono I, Russo P, Bendayan M. 1993. Ultrastructural localization of DNA in immune deposits of human lupus nephritis. *Am J Pathol* 143: 304-11
167. Luo YJ, Tan GZ, Yu M, Li KW, Liu YY, Guo Q, Zeng FQ, Wang L. 2013. Correlation of cutaneous immunoreactants in lesional skin with the serological disorders and disease activity of systemic lupus erythematosus. *PLoS One* 8: e70983
168. Velthuis PJ, Kater L, van der Tweel I, de la Faille HB, van Vloten WA. 1992. Immunofluorescence microscopy of healthy skin from patients with systemic lupus erythematosus: more than just the lupus band. *Ann Rheum Dis* 51: 720-5
169. Satoskar AA, Brodsky SV, Nadasdy G, Bott C, Rovin B, Hebert L, Nadasdy T. 2011. Discrepancies in glomerular and tubulointerstitial/vascular immune complex IgG subclasses in lupus nephritis. *Lupus* 20: 1396-403
170. Kuroki A, Shibata T, Honda H, Totsuka D, Kobayashi K, Sugisaki T. 2002. Glomerular and serum IgG subclasses in diffuse proliferative lupus nephritis, membranous lupus nephritis, and idiopathic membranous nephropathy. *Intern Med* 41: 936-42
171. Izui S, Lambert PH, Miescher PA. 1976. In vitro demonstration of a particular affinity of glomerular basement membrane and collagen for DNA. A possible basis for a local formation of DNA-anti-DNA complexes in systemic lupus erythematosus. *J Exp Med* 144: 428-43
172. Nowling TK, Gilkeson GS. 2011. Mechanisms of tissue injury in lupus nephritis. *Arthritis Res Ther* 13: 250
173. Massengill SF, Goodenow MM, Sleasman JW. 1998. SLE nephritis is associated with an oligoclonal expansion of intrarenal T cells. *Am J Kidney Dis* 31: 418-26
174. Swigris JJ, Fischer A, Gillis J, Meehan RT, Brown KK. 2008. Pulmonary and thrombotic manifestations of systemic lupus erythematosus. *Chest* 133: 271-80
175. Jain D, Halushka MK. 2009. Cardiac pathology of systemic lupus erythematosus. *J Clin Pathol* 62: 584-92
176. Weening JJ, D'Agati VD, Schwartz MM, Seshan SV, Alpers CE, Appel GB, Balow JE, Bruijn JA, Cook T, Ferrario F, Fogo AB, Ginzler EM, Hebert L, Hill G, Hill P, Jennette JC, Kong NC, Lesavre P, Lockshin M, Looi LM, Makino H, Moura LA, Nagata M, International Society of Nephrology Working Group on the Classification of Lupus N, Renal Pathology Society Working Group on the Classification of Lupus N. 2004. The classification of glomerulonephritis in systemic lupus erythematosus revisited. *Kidney Int* 65: 521-30
177. Zecevic RD, Pavlovic MD, Stefanovic D. 2006. Lupus band test and disease activity in systemic lupus erythematosus: does it still matter? *Clin Exp Dermatol* 31: 358-60
178. Sherer Y, Gorstein A, Fritzler MJ, Shoenfeld Y. 2004. Autoantibody explosion in systemic lupus erythematosus: more than 100 different antibodies found in SLE patients. *Semin Arthritis Rheum* 34: 501-37
179. Kallenberg CG. 2008. Anti-C1q autoantibodies. *Autoimmun Rev* 7: 612-5
180. Suzuki M, Hatakeyama A, Kameoka J, Tamate E, Yusa A, Kurosawa K, Saito T, Sasaki T, Yoshinaga K. 1991. Anti-DNA idiotypes deposited in renal glomeruli of patients with lupus nephritis. *Am J Kidney Dis* 18: 232-9

181. Vallin H, Perers A, Alm GV, Ronnblom L. 1999. Anti-double-stranded DNA antibodies and immunostimulatory plasmid DNA in combination mimic the endogenous IFN-alpha inducer in systemic lupus erythematosus. *J Immunol* 163: 6306-13
182. Lovgren T, Eloranta ML, Bave U, Alm GV, Ronnblom L. 2004. Induction of interferon-alpha production in plasmacytoid dendritic cells by immune complexes containing nucleic acid released by necrotic or late apoptotic cells and lupus IgG. *Arthritis Rheum* 50: 1861-72
183. Means TK, Latz E, Hayashi F, Murali MR, Golenbock DT, Luster AD. 2005. Human lupus autoantibody-DNA complexes activate DCs through cooperation of CD32 and TLR9. *J Clin Invest* 115: 407-17
184. Lovgren T, Eloranta ML, Kastner B, Wahren-Herlenius M, Alm GV, Ronnblom L. 2006. Induction of interferon-alpha by immune complexes or liposomes containing systemic lupus erythematosus autoantigen- and Sjogren's syndrome autoantigen-associated RNA. *Arthritis Rheum* 54: 1917-27
185. Boule MW, Broughton C, Mackay F, Akira S, Marshak-Rothstein A, Rifkin IR. 2004. Toll-like receptor 9-dependent and -independent dendritic cell activation by chromatin-immunoglobulin G complexes. *J Exp Med* 199: 1631-40
186. Rifkin IR, Leadbetter EA, Beaudette BC, Kiani C, Monestier M, Shlomchik MJ, Marshak-Rothstein A. 2000. Immune complexes present in the sera of autoimmune mice activate rheumatoid factor B cells. *J Immunol* 165: 1626-33
187. Hahn BH. 1998. Antibodies to DNA. *N Engl J Med* 338: 1359-68
188. ter Borg EJ, Horst G, Hummel EJ, Limburg PC, Kallenberg CG. 1990. Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus. A long-term, prospective study. *Arthritis Rheum* 33: 634-43
189. Amoura Z, Koutouzov S, Chabre H, Cacoub P, Amoura I, Musset L, Bach JF, Piette JC. 2000. Presence of antinucleosome autoantibodies in a restricted set of connective tissue diseases: antinucleosome antibodies of the IgG3 subclass are markers of renal pathogenicity in systemic lupus erythematosus. *Arthritis Rheum* 43: 76-84
190. van Bruggen MC, Walgreen B, Rijke TP, Tamboer W, Kramers K, Smeenk RJ, Monestier M, Fournie GJ, Berden JH. 1997. Antigen specificity of anti-nuclear antibodies complexed to nucleosomes determines glomerular basement membrane binding in vivo. *Eur J Immunol* 27: 1564-9
191. Mostoslavsky G, Fischel R, Yachimovich N, Yarkoni Y, Rosenmann E, Monestier M, Baniyash M, Eilat D. 2001. Lupus anti-DNA autoantibodies cross-react with a glomerular structural protein: a case for tissue injury by molecular mimicry. *Eur J Immunol* 31: 1221-7
192. Ravirajan CT, Rahman MA, Papadaki L, Griffiths MH, Kalsi J, Martin AC, Ehrenstein MR, Latchman DS, Isenberg DA. 1998. Genetic, structural and functional properties of an IgG DNA-binding monoclonal antibody from a lupus patient with nephritis. *Eur J Immunol* 28: 339-50
193. Madaio MP. 2012. The relevance of antigen binding to the pathogenicity of lupus autoantibodies. *Kidney Int* 82: 125-7
194. Ehrenstein MR, Katz DR, Griffiths MH, Papadaki L, Winkler TH, Kalden JR, Isenberg DA. 1995. Human IgG anti-DNA antibodies deposit in kidneys and induce proteinuria in SCID mice. *Kidney Int* 48: 705-11
195. Chan OT, Hannum LG, Haberman AM, Madaio MP, Shlomchik MJ. 1999. A novel mouse with B cells but lacking serum antibody reveals an antibody-independent role for B cells in murine lupus. *J Exp Med* 189: 1639-48
196. Isenberg DA, Manson JJ, Ehrenstein MR, Rahman A. 2007. Fifty years of anti-ds DNA antibodies: are we approaching journey's end? *Rheumatology (Oxford)* 46: 1052-6
197. Pisetsky DS. 2012. The origin and properties of extracellular DNA: from PAMP to DAMP. *Clin Immunol* 144: 32-40

198. Krieg AM. 2002. CpG motifs in bacterial DNA and their immune effects. *Annu Rev Immunol* 20: 709-60
199. Sato Y, Miyata M, Sato Y, Nishimaki T, Kochi H, Kasukawa R. 1999. CpG motif-containing DNA fragments from sera of patients with systemic lupus erythematosus proliferate mononuclear cells in vitro. *J Rheumatol* 26: 294-301
200. Magnusson M, Magnusson S, Vallin H, Ronnblom L, Alm GV. 2001. Importance of CpG dinucleotides in activation of natural IFN-alpha-producing cells by a lupus-related oligodeoxynucleotide. *Scand J Immunol* 54: 543-50
201. Mannik M, Merrill CE, Stamps LD, Wener MH. 2003. Multiple autoantibodies form the glomerular immune deposits in patients with systemic lupus erythematosus. *J Rheumatol* 30: 1495-504
202. Agarwal S, Harper J, Kiely PD. 2009. Concentration of antibodies to extractable nuclear antigens and disease activity in systemic lupus erythematosus. *Lupus* 18: 407-12
203. Riemekasten G, Langnickel D, Ebling FM, Karpouzas G, Kalsi J, Herberth G, Tsao BP, Henklein P, Langer S, Burmester GR, Radbruch A, Hiepe F, Hahn BH. 2003. Identification and characterization of SmD183-119-reactive T cells that provide T cell help for pathogenic anti-double-stranded DNA antibodies. *Arthritis Rheum* 48: 475-85
204. Savarese E, Chae OW, Trowitzsch S, Weber G, Kastner B, Akira S, Wagner H, Schmid RM, Bauer S, Krug A. 2006. U1 small nuclear ribonucleoprotein immune complexes induce type I interferon in plasmacytoid dendritic cells through TLR7. *Blood* 107: 3229-34
205. Lau CM, Broughton C, Tabor AS, Akira S, Flavell RA, Mamula MJ, Christensen SR, Shlomchik MJ, Viglianti GA, Rifkin IR, Marshak-Rothstein A. 2005. RNA-associated autoantigens activate B cells by combined B cell antigen receptor/Toll-like receptor 7 engagement. *J Exp Med* 202: 1171-7
206. Giannakopoulos B, Passam F, Rahgozar S, Krilis SA. 2007. Current concepts on the pathogenesis of the antiphospholipid syndrome. *Blood* 109: 422-30
207. Miyakis S, Lockshin MD, Atsumi T, Branch DW, Brey RL, Cervera R, Derksen RH, PG DEG, Koike T, Meroni PL, Reber G, Shoenfeld Y, Tincani A, Vlachoyiannopoulos PG, Krilis SA. 2006. International consensus statement on an update of the classification criteria for definite antiphospholipid syndrome (APS). *J Thromb Haemost* 4: 295-306
208. Kawakami T, Yamazaki M, Mizoguchi M, Soma Y. 2008. High titer of serum antiphospholipid antibody levels in adult Henoch-Schonlein purpura and cutaneous leukocytoclastic angiitis. *Arthritis Rheum* 59: 561-7
209. Riboldi P, Gerosa M, Raschi E, Testoni C, Meroni PL. 2003. Endothelium as a target for antiphospholipid antibodies. *Immunobiology* 207: 29-36
210. Hurst J, Prinz N, Lorenz M, Bauer S, Chapman J, Lackner KJ, von Landenberg P. 2009. TLR7 and TLR8 ligands and antiphospholipid antibodies show synergistic effects on the induction of IL-1beta and caspase-1 in monocytes and dendritic cells. *Immunobiology* 214: 683-91
211. Haberman AM, William J, Euler C, Shlomchik MJ. 2003. Rheumatoid factors in health and disease: structure, function, induction and regulation. *Curr Dir Autoimmun* 6: 169-95
212. Leadbetter EA, Rifkin IR, Hohlbaum AM, Beaudette BC, Shlomchik MJ, Marshak-Rothstein A. 2002. Chromatin-IgG complexes activate B cells by dual engagement of IgM and Toll-like receptors. *Nature* 416: 603-7
213. Aringer M, Vital E. 2013. Lots of autoantibodies equal lupus? *Arthritis Res Ther* 15: 102
214. Mason LJ, Lambrianides A, Haley JD, Manson JJ, Latchman DS, Isenberg DA, Rahman A. 2005. Stable expression of a recombinant human antinucleosome antibody to investigate relationships between antibody sequence, binding properties, and pathogenicity. *Arthritis Res Ther* 7: R971-83

215. Elkon K, Casali P. 2008. Nature and functions of autoantibodies. *Nat Clin Pract Rheumatol* 4: 491-8
216. Atassi MZ, Casali P. 2008. Molecular mechanisms of autoimmunity. *Autoimmunity* 41: 123-32
217. Olsen NJ, Li QZ, Quan J, Wang L, Mutwally A, Karp DR. 2012. Autoantibody profiling to follow evolution of lupus syndromes. *Arthritis Res Ther* 14: R174
218. Arbuckle MR, James JA, Kohlhase KF, Rubertone MV, Dennis GJ, Harley JB. 2001. Development of anti-dsDNA autoantibodies prior to clinical diagnosis of systemic lupus erythematosus. *Scand J Immunol* 54: 211-9
219. Palsson-McDermott EM, O'Neill LA. 2007. Building an immune system from nine domains. *Biochem Soc Trans* 35: 1437-44
220. Joffre O, Nolte MA, Sporri R, Reis e Sousa C. 2009. Inflammatory signals in dendritic cell activation and the induction of adaptive immunity. *Immunol Rev* 227: 234-47
221. Barr TA, Brown S, Ryan G, Zhao J, Gray D. 2007. TLR-mediated stimulation of APC: Distinct cytokine responses of B cells and dendritic cells. *Eur J Immunol* 37: 3040-53
222. McGettrick AF, O'Neill LA. 2007. Toll-like receptors: key activators of leucocytes and regulator of haematopoiesis. *Br J Haematol* 139: 185-93
223. Patole PS, Grone HJ, Segerer S, Ciubar R, Belemezova E, Henger A, Kretzler M, Schlondorff D, Anders HJ. 2005. Viral double-stranded RNA aggravates lupus nephritis through Toll-like receptor 3 on glomerular mesangial cells and antigen-presenting cells. *J Am Soc Nephrol* 16: 1326-38
224. Marshak-Rothstein A, Rifkin IR. 2007. Immunologically active autoantigens: the role of toll-like receptors in the development of chronic inflammatory disease. *Annu Rev Immunol* 25: 419-41
225. Hornung V, Rothenfusser S, Britsch S, Krug A, Jahrsdorfer B, Giese T, Endres S, Hartmann G. 2002. Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. *J Immunol* 168: 4531-7
226. Peng SL. 2005. Signaling in B cells via Toll-like receptors. *Curr Opin Immunol* 17: 230-6
227. Herlands RA, Christensen SR, Sweet RA, Hershberg U, Shlomchik MJ. 2008. T cell-independent and toll-like receptor-dependent antigen-driven activation of autoreactive B cells. *Immunity* 29: 249-60
228. Hanten JA, Vasilakos JP, Riter CL, Neys L, Lipson KE, Alkan SS, Birmachu W. 2008. Comparison of human B cell activation by TLR7 and TLR9 agonists. *BMC Immunol* 9: 39
229. Christensen SR, Shupe J, Nickerson K, Kashgarian M, Flavell RA, Shlomchik MJ. 2006. Toll-like receptor 7 and TLR9 dictate autoantibody specificity and have opposing inflammatory and regulatory roles in a murine model of lupus. *Immunity* 25: 417-28
230. Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. 2005. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. *Nat Immunol* 6: 769-76
231. Christensen SR, Shlomchik MJ. 2007. Regulation of lupus-related autoantibody production and clinical disease by Toll-like receptors. *Semin Immunol* 19: 11-23
232. Takahashi T, Strober S. 2008. Natural killer T cells and innate immune B cells from lupus-prone NZB/W mice interact to generate IgM and IgG autoantibodies. *Eur J Immunol* 38: 156-65
233. Chavele KM, Ehrenstein MR. 2011. Regulatory T-cells in systemic lupus erythematosus and rheumatoid arthritis. *FEBS Lett* 585: 3603-10
234. Blanco P, Pitard V, Viallard JF, Taupin JL, Pellegrin JL, Moreau JF. 2005. Increase in activated CD8+ T lymphocytes expressing perforin and granzyme B correlates

with disease activity in patients with systemic lupus erythematosus. *Arthritis Rheum* 52: 201-11
235. Yang XY, Wang HY, Zhao XY, Wang LJ, Lv QH, Wang QQ. 2013. Th22, but not Th17 might be a good index to predict the tissue involvement of systemic lupus erythematosus. *J Clin Immunol* 33: 767-74
236. Langer S, Langnickel D, Enghard P, Undeutsch R, Burmester GR, Hiepe F, Radbruch A, Riemekasten G. 2007. The systemic and SmD183-119-autoantigen-specific cytokine memory of Th cells in SLE patients. *Rheumatology (Oxford)* 46: 238-45
237. Desai-Mehta A, Lu L, Ramsey-Goldman R, Datta SK. 1996. Hyperexpression of CD40 ligand by B and T cells in human lupus and its role in pathogenic autoantibody production. *J Clin Invest* 97: 2063-73
238. Wei C, Anolik J, Cappione A, Zheng B, Pugh-Bernard A, Brooks J, Lee EH, Milner EC, Sanz I. 2007. A new population of cells lacking expression of CD27 represents a notable component of the B cell memory compartment in systemic lupus erythematosus. *J Immunol* 178: 6624-33
239. Masutani K, Akahoshi M, Tsuruya K, Tokumoto M, Ninomiya T, Kohsaka T, Fukuda K, Kanai H, Nakashima H, Otsuka T, Hirakata H. 2001. Predominance of Th1 immune response in diffuse proliferative lupus nephritis. *Arthritis Rheum* 44: 2097-106
240. Yang J, Chu Y, Yang X, Gao D, Zhu L, Yang X, Wan L, Li M. 2009. Th17 and natural Treg cell population dynamics in systemic lupus erythematosus. *Arthritis Rheum* 60: 1472-83
241. Blanco P, Palucka AK, Gill M, Pascual V, Banchereau J. 2001. Induction of dendritic cell differentiation by IFN-alpha in systemic lupus erythematosus. *Science* 294: 1540-3
242. Jury EC, Eldridge J, Isenberg DA, Kabouridis PS. 2007. Agrin signalling contributes to cell activation and is overexpressed in T lymphocytes from lupus patients. *J Immunol* 179: 7975-83
243. Yan B, Ye S, Chen G, Kuang M, Shen N, Chen S. 2008. Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. *Arthritis Rheum* 58: 801-12
244. Kiefer K, Oropallo MA, Cancro MP, Marshak-Rothstein A. 2012. Role of type I interferons in the activation of autoreactive B cells. *Immunol Cell Biol* 90: 498-504
245. Ehrenstein MR, Evans JG, Singh A, Moore S, Warnes G, Isenberg DA, Mauri C. 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *J Exp Med* 200: 277-85
246. Annunziato F, Cosmi L, Liotta F, Maggi E, Romagnani S. 2008. The phenotype of human Th17 cells and their precursors, the cytokines that mediate their differentiation and the role of Th17 cells in inflammation. *Int Immunol* 20: 1361-8
247. Fritsch RD, Shen X, Illei GG, Yarboro CH, Prussin C, Hathcock KS, Hodes RJ, Lipsky PE. 2006. Abnormal differentiation of memory T cells in systemic lupus erythematosus. *Arthritis Rheum* 54: 2184-97
248. Gordon C, Matthews N, Schlesinger BC, Akbar AN, Bacon PA, Emery P, Salmon M. 1996. Active systemic lupus erythematosus is associated with the recruitment of naive/resting T cells. *Br J Rheumatol* 35: 226-30
249. Miyara M, Amoura Z, Parizot C, Badoual C, Dorgham K, Trad S, Nochy D, Debre P, Piette JC, Gorochov G. 2005. Global natural regulatory T cell depletion in active systemic lupus erythematosus. *J Immunol* 175: 8392-400
250. Valencia X, Yarboro C, Illei G, Lipsky PE. 2007. Deficient CD4+CD25high T regulatory cell function in patients with active systemic lupus erythematosus. *J Immunol* 178: 2579-88
251. Blair PA, Norena LY, Flores-Borja F, Rawlings DJ, Isenberg DA, Ehrenstein MR, Mauri C. 2010. CD19(+)CD24(hi)CD38(hi) B cells exhibit regulatory capacity in healthy individuals but are functionally impaired in systemic Lupus Erythematosus patients. *Immunity* 32: 129-40

252. Hahn BH, Ebling F, Singh RR, Singh RP, Karpouzas G, La Cava A. 2005. Cellular and molecular mechanisms of regulation of autoantibody production in lupus. *Ann N Y Acad Sci* 1051: 433-41
253. Hsu WT, Suen JL, Chiang BL. 2006. The role of CD4CD25 T cells in autoantibody production in murine lupus. *Clin Exp Immunol* 145: 513-9
254. Wu HY, Quintana FJ, Weiner HL. 2008. Nasal anti-CD3 antibody ameliorates lupus by inducing an IL-10-secreting CD4+ CD25- LAP+ regulatory T cell and is associated with down-regulation of IL-17+ CD4+ ICOS+ CXCR5+ follicular helper T cells. *J Immunol* 181: 6038-50
255. Lee JH, Wang LC, Lin YT, Yang YH, Lin DT, Chiang BL. 2006. Inverse correlation between CD4+ regulatory T-cell population and autoantibody levels in paediatric patients with systemic lupus erythematosus. *Immunology* 117: 280-6
256. Chan OT, Madaio MP, Shlomchik MJ. 1999. The central and multiple roles of B cells in lupus pathogenesis. *Immunol Rev* 169: 107-21
257. von Boehmer H, Melchers F. 2010. Checkpoints in lymphocyte development and autoimmune disease. *Nat Immunol* 11: 14-20
258. Wardemann H, Yurasov S, Schaefer A, Young JW, Meffre E, Nussenzweig MC. 2003. Predominant autoantibody production by early human B cell precursors. *Science* 301: 1374-7
259. Tsuiji M, Yurasov S, Velinzon K, Thomas S, Nussenzweig MC, Wardemann H. 2006. A checkpoint for autoreactivity in human IgM+ memory B cell development. *J Exp Med* 203: 393-400
260. Halverson R, Torres RM, Pelanda R. 2004. Receptor editing is the main mechanism of B cell tolerance toward membrane antigens. *Nat Immunol* 5: 645-50
261. Yurasov S, Wardemann H, Hammersen J, Tsuiji M, Meffre E, Pascual V, Nussenzweig MC. 2005. Defective B cell tolerance checkpoints in systemic lupus erythematosus. *J Exp Med* 201: 703-11
262. Zhang J, Jacobi AM, Wang T, Diamond B. 2008. Pathogenic autoantibodies in systemic lupus erythematosus are derived from both self-reactive and non-self-reactive B cells. *Mol Med* 14: 675-81
263. Jacobi AM, Zhang J, Mackay M, Aranow C, Diamond B. 2009. Phenotypic characterization of autoreactive B cells--checkpoints of B cell tolerance in patients with systemic lupus erythematosus. *PLoS One* 4: e5776
264. Cappione A, 3rd, Anolik JH, Pugh-Bernard A, Barnard J, Dutcher P, Silverman G, Sanz I. 2005. Germinal center exclusion of autoreactive B cells is defective in human systemic lupus erythematosus. *J Clin Invest* 115: 3205-16
265. Hutloff A, Buchner K, Reiter K, Baelde HJ, Odendahl M, Jacobi A, Dorner T, Kroczek RA. 2004. Involvement of inducible costimulator in the exaggerated memory B cell and plasma cell generation in systemic lupus erythematosus. *Arthritis Rheum* 50: 3211-20
266. Pugh-Bernard AE, Silverman GJ, Cappione AJ, Villano ME, Ryan DH, Insel RA, Sanz I. 2001. Regulation of inherently autoreactive VH4-34 B cells in the maintenance of human B cell tolerance. *J Clin Invest* 108: 1061-70
267. Morimoto S, Nakano S, Watanabe T, Tamayama Y, Mitsuo A, Nakiri Y, Suzuki J, Nozawa K, Amano H, Tokano Y, Kobata T, Takasaki Y. 2007. Expression of B-cell activating factor of the tumour necrosis factor family (BAFF) in T cells in active systemic lupus erythematosus: the role of BAFF in T cell-dependent B cell pathogenic autoantibody production. *Rheumatology (Oxford)* 46: 1083-6
268. Kikuchi Y, Koarada S, Tada Y, Ushiyama O, Morito F, Suzuki N, Ohta A, Miyake K, Kimoto M, Horiuchi T, Nagasawa K. 2002. RP105-lacking B cells from lupus patients are responsible for the production of immunoglobulins and autoantibodies. *Arthritis Rheum* 46: 3259-65
269. Divanovic S, Trompette A, Atabani SF, Madan R, Golenbock DT, Visintin A, Finberg RW, Tarakhovsky A, Vogel SN, Belkaid Y, Kurt-Jones EA, Karp CL. 2005.

Negative regulation of Toll-like receptor 4 signaling by the Toll-like receptor homolog RP105. *Nat Immunol* 6: 571-8
270. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. 2005. Identification and characterization of circulating human transitional B cells. *Blood* 105: 4390-8
271. Jacobi AM, Reiter K, Mackay M, Aranow C, Hiepe F, Radbruch A, Hansen A, Burmester GR, Diamond B, Lipsky PE, Dorner T. 2008. Activated memory B cell subsets correlate with disease activity in systemic lupus erythematosus: delineation by expression of CD27, IgD, and CD95. *Arthritis Rheum* 58: 1762-73
272. Rodriguez-Bayona B, Ramos-Amaya A, Perez-Venegas JJ, Rodriguez C, Brieva JA. 2010. Decreased frequency and activated phenotype of blood CD27 IgD IgM B lymphocytes is a permanent abnormality in systemic lupus erythematosus patients. *Arthritis Res Ther* 12: R108
273. Nicholas MW, Dooley MA, Hogan SL, Anolik J, Looney J, Sanz I, Clarke SH. 2008. A novel subset of memory B cells is enriched in autoreactivity and correlates with adverse outcomes in SLE. *Clin Immunol* 126: 189-201
274. Taher TE, Parikh K, Flores-Borja F, Mletzko S, Isenberg DA, Peppelenbosch MP, Mageed RA. 2010. Protein phosphorylation and kinome profiling reveal altered regulation of multiple signaling pathways in B lymphocytes from patients with systemic lupus erythematosus. *Arthritis Rheum* 62: 2412-23
275. Kono H, Kyogoku C, Suzuki T, Tsuchiya N, Honda H, Yamamoto K, Tokunaga K, Honda Z. 2005. FcgammaRIIB Ile232Thr transmembrane polymorphism associated with human systemic lupus erythematosus decreases affinity to lipid rafts and attenuates inhibitory effects on B cell receptor signaling. *Hum Mol Genet* 14: 2881-92
276. Manjarrez-Orduno N, Marasco E, Chung SA, Katz MS, Kiridly JF, Simpfendorfer KR, Freudenberg J, Ballard DH, Nashi E, Hopkins TJ, Cunninghame Graham DS, Lee AT, Coenen MJ, Franke B, Swinkels DW, Graham RR, Kimberly RP, Gaffney PM, Vyse TJ, Behrens TW, Criswell LA, Diamond B, Gregersen PK. 2012. CSK regulatory polymorphism is associated with systemic lupus erythematosus and influences B-cell signaling and activation. *Nat Genet* 44: 1227-30
277. Sanz I, Wei C, Lee FE, Anolik J. 2008. Phenotypic and functional heterogeneity of human memory B cells. *Semin Immunol* 20: 67-82
278. Llorente L, Richaud-Patin Y, Fior R, Alcocer-Varela J, Wijdenes J, Fourrier BM, Galanaud P, Emilie D. 1994. In vivo production of interleukin-10 by non-T cells in rheumatoid arthritis, Sjogren's syndrome, and systemic lupus erythematosus. A potential mechanism of B lymphocyte hyperactivity and autoimmunity. *Arthritis Rheum* 37: 1647-55
279. Llorente L, Zou W, Levy Y, Richaud-Patin Y, Wijdenes J, Alcocer-Varela J, Morel-Fourrier B, Brouet JC, Alarcon-Segovia D, Galanaud P, Emilie D. 1995. Role of interleukin 10 in the B lymphocyte hyperactivity and autoantibody production of human systemic lupus erythematosus. *J Exp Med* 181: 839-44
280. Kitani A, Hara M, Hirose T, Harigai M, Suzuki K, Kawakami M, Kawaguchi Y, Hidaka T, Kawagoe M, Nakamura H. 1992. Autostimulatory effects of IL-6 on excessive B cell differentiation in patients with systemic lupus erythematosus: analysis of IL-6 production and IL-6R expression. *Clin Exp Immunol* 88: 75-83
281. Linton PJ, Harbertson J, Bradley LM. 2000. A critical role for B cells in the development of memory CD4 cells. *J Immunol* 165: 5558-65
282. Shah S, Qiao L. 2008. Resting B cells expand a CD4+CD25+Foxp3+ Treg population via TGF-beta3. *Eur J Immunol* 38: 2488-98
283. Tretter T, Venigalla RK, Eckstein V, Saffrich R, Sertel S, Ho AD, Lorenz HM. 2008. Induction of CD4+ T-cell anergy and apoptosis by activated human B cells. *Blood* 112: 4555-64
284. Arens R, Tesselaar K, Baars PA, van Schijndel GM, Hendriks J, Pals ST, Krimpenfort P, Borst J, van Oers MH, van Lier RA. 2001. Constitutive CD27/CD70

interaction induces expansion of effector-type T cells and results in IFNgamma-mediated B cell depletion. *Immunity* 15: 801-12
285. Tesselaar K, Arens R, van Schijndel GM, Baars PA, van der Valk MA, Borst J, van Oers MH, van Lier RA. 2003. Lethal T cell immunodeficiency induced by chronic costimulation via CD27-CD70 interactions. *Nat Immunol* 4: 49-54
286. Liu Z, Zou Y, Davidson A. 2011. Plasma cells in systemic lupus erythematosus: the long and short of it all. *Eur J Immunol* 41: 588-91
287. Jacobi AM, Mei H, Hoyer BF, Mumtaz IM, Thiele K, Radbruch A, Burmester GR, Hiepe F, Dorner T. 2010. HLA-DRhigh/CD27high plasmablasts indicate active disease in patients with systemic lupus erythematosus. *Ann Rheum Dis* 69: 305-8
288. Kawamoto M, Harigai M, Hara M, Kawaguchi Y, Tezuka K, Tanaka M, Sugiura T, Katsumata Y, Fukasawa C, Ichida H, Higami S, Kamatani N. 2006. Expression and function of inducible co-stimulator in patients with systemic lupus erythematosus: possible involvement in excessive interferon-gamma and anti-double-stranded DNA antibody production. *Arthritis Res Ther* 8: R62
289. Chan O, Shlomchik MJ. 1998. A new role for B cells in systemic autoimmunity: B cells promote spontaneous T cell activation in MRL-lpr/lpr mice. *J Immunol* 160: 51-9
290. Sfikakis PP, Boletis JN, Lionaki S, Vigklis V, Fragiadaki KG, Iniotaki A, Moutsopoulos HM. 2005. Remission of proliferative lupus nephritis following B cell depletion therapy is preceded by down-regulation of the T cell costimulatory molecule CD40 ligand: an open-label trial. *Arthritis Rheum* 52: 501-13
291. Yan J, Harvey BP, Gee RJ, Shlomchik MJ, Mamula MJ. 2006. B cells drive early T cell autoimmunity in vivo prior to dendritic cell-mediated autoantigen presentation. *J Immunol* 177: 4481-7
292. Vallerskog T, Gunnarsson I, Widhe M, Risselada A, Klareskog L, van Vollenhoven R, Malmstrom V, Trollmo C. 2007. Treatment with rituximab affects both the cellular and the humoral arm of the immune system in patients with SLE. *Clin Immunol* 122: 62-74
293. Vigna-Perez M, Hernandez-Castro B, Paredes-Saharopulos O, Portales-Perez D, Baranda L, Abud-Mendoza C, Gonzalez-Amaro R. 2006. Clinical and immunological effects of Rituximab in patients with lupus nephritis refractory to conventional therapy: a pilot study. *Arthritis Res Ther* 8: R83
294. Tokunaga M, Saito K, Kawabata D, Imura Y, Fujii T, Nakayamada S, Tsujimura S, Nawata M, Iwata S, Azuma T, Mimori T, Tanaka Y. 2007. Efficacy of rituximab (anti-CD20) for refractory systemic lupus erythematosus involving the central nervous system. *Ann Rheum Dis* 66: 470-5
295. Reis EA, Athanazio DA, Lima I, NO ES, Andrade JC, Jesus RN, Barbosa LM, Reis MG, Santiago MB. 2009. NK and NKT cell dynamics after rituximab therapy for systemic lupus erythematosus and rheumatoid arthritis. *Rheumatol Int* 29: 469-75
296. Crispin JC, Martinez A, de Pablo P, Velasquillo C, Alcocer-Varela J. 1998. Participation of the CD69 antigen in the T-cell activation process of patients with systemic lupus erythematosus. *Scand J Immunol* 48: 196-200
297. Wouters CH, Diegenant C, Ceuppens JL, Degreef H, Stevens EA. 2004. The circulating lymphocyte profiles in patients with discoid lupus erythematosus and systemic lupus erythematosus suggest a pathogenetic relationship. *Br J Dermatol* 150: 693-700
298. Moulton VR, Tsokos GC. 2011. Abnormalities of T cell signaling in systemic lupus erythematosus. *Arthritis Res Ther* 13: 207
299. Prinz JC, Meurer M, Reiter C, Rieber EP, Plewig G, Riethmuller G. 1996. Treatment of severe cutaneous lupus erythematosus with a chimeric CD4 monoclonal antibody, cM-T412. *J Am Acad Dermatol* 34: 244-52
300. Riemekasten G, Kawald A, Weiss C, Meine A, Marell J, Klein R, Hocher B, Meisel C, Hausdorf G, Manz R, Kamradt T, Burmester GR, Hiepe F. 2001. Strong

acceleration of murine lupus by injection of the SmD1(83-119) peptide. *Arthritis Rheum* 44: 2435-45
301. Lu L, Kaliyaperumal A, Boumpas DT, Datta SK. 1999. Major peptide autoepitopes for nucleosome-specific T cells of human lupus. *J Clin Invest* 104: 345-55
302. Greidinger EL, Gazitt T, Jaimes KF, Hoffman RW. 2004. Human T cell clones specific for heterogeneous nuclear ribonucleoprotein A2 autoantigen from connective tissue disease patients assist in autoantibody production. *Arthritis Rheum* 50: 2216-22
303. Linterman MA, Rigby RJ, Wong RK, Yu D, Brink R, Cannons JL, Schwartzberg PL, Cook MC, Walters GD, Vinuesa CG. 2009. Follicular helper T cells are required for systemic autoimmunity. *J Exp Med* 206: 561-76
304. Simpson N, Gatenby PA, Wilson A, Malik S, Fulcher DA, Tangye SG, Manku H, Vyse TJ, Roncador G, Huttley GA, Goodnow CC, Vinuesa CG, Cook MC. 2010. Expansion of circulating T cells resembling follicular helper T cells is a fixed phenotype that identifies a subset of severe systemic lupus erythematosus. *Arthritis Rheum* 62: 234-44
305. Reininger L, Radaszkiewicz T, Kosco M, Melchers F, Rolink AG. 1992. Development of autoimmune disease in SCID mice populated with long-term "in vitro" proliferating (NZB x NZW)F1 pre-B cells. *J Exp Med* 176: 1343-53
306. Wen L, Roberts SJ, Viney JL, Wong FS, Mallick C, Findly RC, Peng Q, Craft JE, Owen MJ, Hayday AC. 1994. Immunoglobulin synthesis and generalized autoimmunity in mice congenitally deficient in alpha beta(+) T cells. *Nature* 369: 654-8
307. Kato T, Kurokawa M, Sasakawa H, Masuko-Hongo K, Matsui T, Sekine T, Tanaka C, Yamamoto K, Nishioka K. 2000. Analysis of accumulated T cell clonotypes in patients with systemic lupus erythematosus. *Arthritis Rheum* 43: 2712-21
308. Dolff S, Abdulahad WH, van Dijk MC, Limburg PC, Kallenberg CG, Bijl M. 2010. Urinary T cells in active lupus nephritis show an effector memory phenotype. *Ann Rheum Dis* 69: 2034-41
309. Calvani N, Richards HB, Tucci M, Pannarale G, Silvestris F. 2004. Up-regulation of IL-18 and predominance of a Th1 immune response is a hallmark of lupus nephritis. *Clin Exp Immunol* 138: 171-8
310. Wong CK, Lit LC, Tam LS, Li EK, Wong PT, Lam CW. 2008. Hyperproduction of IL-23 and IL-17 in patients with systemic lupus erythematosus: implications for Th17-mediated inflammation in auto-immunity. *Clin Immunol* 127: 385-93
311. Tucci M, Lombardi L, Richards HB, Dammacco F, Silvestris F. 2008. Overexpression of interleukin-12 and T helper 1 predominance in lupus nephritis. *Clin Exp Immunol* 154: 247-54
312. Funauchi M, Ikoma S, Enomoto H, Horiuchi A. 1998. Decreased Th1-like and increased Th2-like cells in systemic lupus erythematosus. *Scand J Rheumatol* 27: 219-24
313. Akahoshi M, Nakashima H, Tanaka Y, Kohsaka T, Nagano S, Ohgami E, Arinobu Y, Yamaoka K, Niiro H, Shinozaki M, Hirakata H, Horiuchi T, Otsuka T, Niho Y. 1999. Th1/Th2 balance of peripheral T helper cells in systemic lupus erythematosus. *Arthritis Rheum* 42: 1644-8
314. Chang DM, Su WL, Chu SJ. 2002. The expression and significance of intracellular T helper cytokines in systemic lupus erythematosus. *Immunol Invest* 31: 1-12
315. Miyake K, Akahoshi M, Nakashima H. 2011. Th subset balance in lupus nephritis. *J Biomed Biotechnol* 2011: 980286
316. Lu XY, Zhu CQ, Qian J, Chen XX, Ye S, Gu YY. 2010. Intrathecal cytokine and chemokine profiling in neuropsychiatric lupus or lupus complicated with central nervous system infection. *Lupus* 19: 689-95
317. Segal R, Bermas BL, Dayan M, Kalush F, Shearer GM, Mozes E. 1997. Kinetics of cytokine production in experimental systemic lupus erythematosus: involvement of T helper cell 1/T helper cell 2-type cytokines in disease. *J Immunol* 158: 3009-16

318. Horwitz DA, Gray JD, Behrendsen SC, Kubin M, Rengaraju M, Ohtsuka K, Trinchieri G. 1998. Decreased production of interleukin-12 and other Th1-type cytokines in patients with recent-onset systemic lupus erythematosus. *Arthritis Rheum* 41: 838-44
319. Szodoray P, Nakken B, Barath S, Csipo I, Nagy G, El-Hage F, Osnes LT, Szegedi G, Bodolay E. 2013. Altered Th17 cells and Th17/regulatory T-cell ratios indicate the subsequent conversion from undifferentiated connective tissue disease to definitive systemic autoimmune disorders. *Hum Immunol* 74: 1510-8
320. Yamada M, Yagita H, Inoue H, Takanashi T, Matsuda H, Munechika E, Kanamaru Y, Shirato I, Tomino Y, Matushima K, Okumura K, Hashimoto H. 2002. Selective accumulation of CCR4+ T lymphocytes into renal tissue of patients with lupus nephritis. *Arthritis Rheum* 46: 735-40
321. Segerer S, Banas B, Wornle M, Schmid H, Cohen CD, Kretzler M, Mack M, Kiss E, Nelson PJ, Schlondorff D, Grone HJ. 2004. CXCR3 is involved in tubulointerstitial injury in human glomerulonephritis. *Am J Pathol* 164: 635-49
322. Wenzel J, Henze S, Worenkamper E, Basner-Tschakarjan E, Sokolowska-Wojdylo M, Steitz J, Bieber T, Tuting T. 2005. Role of the chemokine receptor CCR4 and its ligand thymus- and activation-regulated chemokine/CCL17 for lymphocyte recruitment in cutaneous lupus erythematosus. *J Invest Dermatol* 124: 1241-8
323. Wenzel J, Zahn S, Mikus S, Wiechert A, Bieber T, Tuting T. 2007. The expression pattern of interferon-inducible proteins reflects the characteristic histological distribution of infiltrating immune cells in different cutaneous lupus erythematosus subsets. *Br J Dermatol* 157: 752-7
324. Freutel S, Gaffal E, Zahn S, Bieber T, Tuting T, Wenzel J. 2011. Enhanced CCR5+/CCR3+ T helper cell ratio in patients with active cutaneous lupus erythematosus. *Lupus* 20: 1300-4
325. Sakaguchi S, Yamaguchi T, Nomura T, Ono M. 2008. Regulatory T cells and immune tolerance. *Cell* 133: 775-87
326. Larche M. 2007. Regulatory T cells in allergy and asthma. *Chest* 132: 1007-14
327. Zhang B, Zhang X, Tang FL, Zhu LP, Liu Y, Lipsky PE. 2008. Clinical significance of increased CD4+CD25-Foxp3+ T cells in patients with new-onset systemic lupus erythematosus. *Ann Rheum Dis* 67: 1037-40
328. Bonelli M, Savitskaya A, Steiner CW, Rath E, Smolen JS, Scheinecker C. 2009. Phenotypic and functional analysis of CD4+ CD25- Foxp3+ T cells in patients with systemic lupus erythematosus. *J Immunol* 182: 1689-95
329. Venigalla RK, Tretter T, Krienke S, Max R, Eckstein V, Blank N, Fiehn C, Ho AD, Lorenz HM. 2008. Reduced CD4+,CD25- T cell sensitivity to the suppressive function of CD4+,CD25high,CD127 -/low regulatory T cells in patients with active systemic lupus erythematosus. *Arthritis Rheum* 58: 2120-30
330. Parietti V, Monneaux F, Decossas M, Muller S. 2008. Function of CD4+,CD25+ Treg cells in MRL/lpr mice is compromised by intrinsic defects in antigen-presenting cells and effector T cells. *Arthritis Rheum* 58: 1751-61
331. Jury EC, Flores-Borja F, Kalsi HS, Lazarus M, Isenberg DA, Mauri C, Ehrenstein MR. 2010. Abnormal CTLA-4 function in T cells from patients with systemic lupus erythematosus. *Eur J Immunol* 40: 569-78
332. Kuhn A, Beissert S, Krammer PH. 2009. CD4(+)CD25 (+) regulatory T cells in human lupus erythematosus. *Arch Dermatol Res* 301: 71-81
333. Lee HY, Hong YK, Yun HJ, Kim YM, Kim JR, Yoo WH. 2008. Altered frequency and migration capacity of CD4+CD25+ regulatory T cells in systemic lupus erythematosus. *Rheumatology (Oxford)* 47: 789-94
334. Cuda CM, Wan S, Sobel ES, Croker BP, Morel L. 2007. Murine lupus susceptibility locus Sle1a controls regulatory T cell number and function through multiple mechanisms. *J Immunol* 179: 7439-47
335. Matzinger P. 2002. The danger model: a renewed sense of self. *Science* 296: 301-5

336. Tian J, Avalos AM, Mao SY, Chen B, Senthil K, Wu H, Parroche P, Drabic S, Golenbock D, Sirois C, Hua J, An LL, Audoly L, La Rosa G, Bierhaus A, Naworth P, Marshak-Rothstein A, Crow MK, Fitzgerald KA, Latz E, Kiener PA, Coyle AJ. 2007. Toll-like receptor 9-dependent activation by DNA-containing immune complexes is mediated by HMGB1 and RAGE. *Nat Immunol* 8: 487-96
337. Isenberg DA, Patterson KG, Todd-Pokropek A, Snaith ML, Goldstone AH. 1982. Haematological aspects of systemic lupus erythematosus: a reappraisal using automated methods. *Acta Haematol* 67: 242-8
338. Fries LF, Mullins WW, Cho KR, Plotz PH, Frank MM. 1984. Monocyte receptors for the Fc portion of IgG are increased in systemic lupus erythematosus. *J Immunol* 132: 695-700
339. Li Y, Lee PY, Sobel ES, Narain S, Satoh M, Segal MS, Reeves WH, Richards HB. 2009. Increased expression of FcgammaRI/CD64 on circulating monocytes parallels ongoing inflammation and nephritis in lupus. *Arthritis Res Ther* 11: R6
340. Katsiari CG, Liossis SN, Souliotis VL, Dimopoulos AM, Manoussakis MN, Sfikakis PP. 2002. Aberrant expression of the costimulatory molecule CD40 ligand on monocytes from patients with systemic lupus erythematosus. *Clin Immunol* 103: 54-62
341. Jin O, Kavikondala S, Sun L, Fu R, Mok MY, Chan A, Yeung J, Lau CS. 2008. Systemic lupus erythematosus patients have increased number of circulating plasmacytoid dendritic cells, but decreased myeloid dendritic cells with deficient CD83 expression. *Lupus* 17: 654-62
342. Ding D, Mehta H, McCune WJ, Kaplan MJ. 2006. Aberrant phenotype and function of myeloid dendritic cells in systemic lupus erythematosus. *J Immunol* 177: 5878-89
343. Carreno LJ, Pacheco R, Gutierrez MA, Jacobelli S, Kalergis AM. 2009. Disease activity in systemic lupus erythematosus is associated with an altered expression of low-affinity Fc gamma receptors and costimulatory molecules on dendritic cells. *Immunology* 128: 334-41
344. Decker P, Singh-Jasuja H, Haager S, Kotter I, Rammensee HG. 2005. Nucleosome, the main autoantigen in systemic lupus erythematosus, induces direct dendritic cell activation via a MyD88-independent pathway: consequences on inflammation. *J Immunol* 174: 3326-34
345. Urbonaviciute V, Furnrohr BG, Meister S, Munoz L, Heyder P, De Marchis F, Bianchi ME, Kirschning C, Wagner H, Manfredi AA, Kalden JR, Schett G, Rovere-Querini P, Herrmann M, Voll RE. 2008. Induction of inflammatory and immune responses by HMGB1-nucleosome complexes: implications for the pathogenesis of SLE. *J Exp Med* 205: 3007-18
346. Joo H, Coquery C, Xue Y, Gayet I, Dillon SR, Punaro M, Zurawski G, Banchereau J, Pascual V, Oh S. 2012. Serum from patients with SLE instructs monocytes to promote IgG and IgA plasmablast differentiation. *J Exp Med* 209: 1335-48
347. Dai C, Liu Z, Zhou H, Li L. 2001. Monocyte chemoattractant protein-1 expression in renal tissue is associated with monocyte recruitment and tubulo-interstitial lesions in patients with lupus nephritis. *Chin Med J (Engl)* 114: 864-8
348. Marks SD, Williams SJ, Tullus K, Sebire NJ. 2008. Glomerular expression of monocyte chemoattractant protein-1 is predictive of poor renal prognosis in pediatric lupus nephritis. *Nephrol Dial Transplant* 23: 3521-6
349. Hanayama R, Tanaka M, Miyasaka K, Aozasa K, Koike M, Uchiyama Y, Nagata S. 2004. Autoimmune disease and impaired uptake of apoptotic cells in MFG-E8-deficient mice. *Science* 304: 1147-50
350. Ren Y, Tang J, Mok MY, Chan AW, Wu A, Lau CS. 2003. Increased apoptotic neutrophils and macrophages and impaired macrophage phagocytic clearance of apoptotic neutrophils in systemic lupus erythematosus. *Arthritis Rheum* 48: 2888-97
351. Tas SW, Quartier P, Botto M, Fossati-Jimack L. 2006. Macrophages from patients with SLE and rheumatoid arthritis have defective adhesion in vitro, while only SLE macrophages have impaired uptake of apoptotic cells. *Ann Rheum Dis* 65: 216-21

352. Shao WH, Cohen PL. 2011. Disturbances of apoptotic cell clearance in systemic lupus erythematosus. *Arthritis Res Ther* 13: 202
353. Yamaguchi H, Fujimoto T, Nakamura S, Ohmura K, Mimori T, Matsuda F, Nagata S. 2010. Aberrant splicing of the milk fat globule-EGF factor 8 (MFG-E8) gene in human systemic lupus erythematosus. *Eur J Immunol* 40: 1778-85
354. Palucka AK, Banchereau J, Blanco P, Pascual V. 2002. The interplay of dendritic cell subsets in systemic lupus erythematosus. *Immunol Cell Biol* 80: 484-8
355. Sacre K, Criswell LA, McCune JM. 2012. Hydroxychloroquine is associated with impaired interferon-alpha and tumor necrosis factor-alpha production by plasmacytoid dendritic cells in systemic lupus erythematosus. *Arthritis Res Ther* 14: R155
356. Cella M, Jarrossay D, Facchetti F, Alebardi O, Nakajima H, Lanzavecchia A, Colonna M. 1999. Plasmacytoid monocytes migrate to inflamed lymph nodes and produce large amounts of type I interferon. *Nat Med* 5: 919-23
357. Farkas L, Beiske K, Lund-Johansen F, Brandtzaeg P, Jahnsen FL. 2001. Plasmacytoid dendritic cells (natural interferon- alpha/beta-producing cells) accumulate in cutaneous lupus erythematosus lesions. *Am J Pathol* 159: 237-43
358. Wenzel J, Worenkamper E, Freutel S, Henze S, Haller O, Bieber T, Tuting T. 2005. Enhanced type I interferon signalling promotes Th1-biased inflammation in cutaneous lupus erythematosus. *J Pathol* 205: 435-42
359. Tucci M, Quatraro C, Lombardi L, Pellegrino C, Dammacco F, Silvestris F. 2008. Glomerular accumulation of plasmacytoid dendritic cells in active lupus nephritis: role of interleukin-18. *Arthritis Rheum* 58: 251-62
360. Vermi W, Lonardi S, Morassi M, Rossini C, Tardanico R, Venturini M, Sala R, Tincani A, Poliani PL, Calzavara-Pinton PG, Cerroni L, Santoro A, Facchetti F. 2009. Cutaneous distribution of plasmacytoid dendritic cells in lupus erythematosus. Selective tropism at the site of epithelial apoptotic damage. *Immunobiology* 214: 877-86
361. Thacker SG, Berthier CC, Mattinzoli D, Rastaldi MP, Kretzler M, Kaplan MJ. 2010. The detrimental effects of IFN-alpha on vasculogenesis in lupus are mediated by repression of IL-1 pathways: potential role in atherogenesis and renal vascular rarefaction. *J Immunol* 185: 4457-69
362. Molad Y, Buyon J, Anderson DC, Abramson SB, Cronstein BN. 1994. Intravascular neutrophil activation in systemic lupus erythematosus (SLE): dissociation between increased expression of CD11b/CD18 and diminished expression of L-selectin on neutrophils from patients with active SLE. *Clin Immunol Immunopathol* 71: 281-6
363. Courtney PA, Crockard AD, Williamson K, Irvine AE, Kennedy RJ, Bell AL. 1999. Increased apoptotic peripheral blood neutrophils in systemic lupus erythematosus: relations with disease activity, antibodies to double stranded DNA, and neutropenia. *Ann Rheum Dis* 58: 309-14
364. Larson AR, Granter SR. 2014. Systemic lupus erythematosus-associated neutrophilic dermatosis: a review and update. *Adv Anat Pathol* 21: 248-53
365. Villanueva E, Yalavarthi S, Berthier CC, Hodgin JB, Khandpur R, Lin AM, Rubin CJ, Zhao W, Olsen SH, Klinker M, Shealy D, Denny MF, Plumas J, Chaperot L, Kretzler M, Bruce AT, Kaplan MJ. 2011. Netting neutrophils induce endothelial damage, infiltrate tissues, and expose immunostimulatory molecules in systemic lupus erythematosus. *J Immunol* 187: 538-52
366. Charles N, Hardwick D, Daugas E, Illei GG, Rivera J. 2010. Basophils and the T helper 2 environment can promote the development of lupus nephritis. *Nat Med* 16: 701-7
367. Liang P, Tang Y, Fu S, Lv J, Liu B, Feng M, Li J, Lai D, Wan X, Xu A. 2014. Basophil count, a marker for disease activity in systemic lupus erythematosus. *Clin Rheumatol*
368. Vila LM, Alarcon GS, McGwin G, Jr., Bastian HM, Fessler BJ, Reveille JD, Lumina Study G. 2006. Systemic lupus erythematosus in a multiethnic US cohort, XXXVII:

association of lymphopenia with clinical manifestations, serologic abnormalities, disease activity, and damage accrual. *Arthritis Rheum* 55: 799-806
369. Beyan E, Beyan C, Turan M. 2007. Hematological presentation in systemic lupus erythematosus and its relationship with disease activity. *Hematology* 12: 257-61
370. Utsinger PD. 1976. Relationship of lymphocytotoxic antibodies to lymphopenia and parameters of disease activity in systemic lupus erythematosus. *J Rheumatol* 3: 175-85
371. Harley JB, Sestak AL, Willis LG, Fu SM, Hansen JA, Reichlin M. 1989. A model for disease heterogeneity in systemic lupus erythematosus. Relationships between histocompatibility antigens, autoantibodies, and lymphopenia or renal disease. *Arthritis Rheum* 32: 826-36
372. Wenzel J, Gerdsen R, Uerlich M, Bauer R, Tueting T, Bieber T. 2004. Lymphocytopenia in lupus erythematosus: close in vivo association to autoantibodies targeting nuclear antigens. *Br J Dermatol* 150: 994-8
373. Odendahl M, Jacobi A, Hansen A, Feist E, Hiepe F, Burmester GR, Lipsky PE, Radbruch A, Dorner T. 2000. Disturbed peripheral B lymphocyte homeostasis in systemic lupus erythematosus. *J Immunol* 165: 5970-9
374. Robak E, Smolewski P, Wozniacka A, Sysa-Jedrzejowska A, Robak T. 2004. Clinical significance of circulating dendritic cells in patients with systemic lupus erythematosus. *Mediators Inflamm* 13: 171-80
375. Shoenfeld Y, Zamir R, Joshua H, Lavie G, Pinkhas J. 1985. Human monoclonal anti-DNA antibodies react as lymphocytotoxic antibodies. *Eur J Immunol* 15: 1024-8
376. Lenert P, Lenert G, Senecal JL. 1996. CD4-reactive antibodies in systemic lupus erythematosus. *Hum Immunol* 49: 38-48
377. Georgescu L, Vakkalanka RK, Elkon KB, Crow MK. 1997. Interleukin-10 promotes activation-induced cell death of SLE lymphocytes mediated by Fas ligand. *J Clin Invest* 100: 2622-33
378. Silvestris F, Grinello D, Tucci M, Cafforio P, Dammacco F. 2003. Enhancement of T cell apoptosis correlates with increased serum levels of soluble Fas (CD95/Apo-1) in active lupus. *Lupus* 12: 8-14
379. Habib HM, Taher TE, Isenberg DA, Mageed RA. 2008. Enhanced propensity of T lymphocytes in patients with systemic lupus erythematosus to apoptosis in the presence of tumour necrosis factor alpha. *Scand J Rheumatol*: 1-9
380. Ortaldo JR, Mason AT, O'Shea JJ. 1995. Receptor-induced death in human natural killer cells: involvement of CD16. *J Exp Med* 181: 339-44
381. Krammer PH, Arnold R, Lavrik IN. 2007. Life and death in peripheral T cells. *Nat Rev Immunol* 7: 532-42
382. Tagoe C, Shah A, Yee H, Belmont HM. 2001. Aplastic anemia in systemic lupus erythematosus: a distinct presentation of acquired aplastic anemia? *J Clin Rheumatol* 7: 377-83
383. Lin Q, Dong C, Cooper MD. 1998. Impairment of T and B cell development by treatment with a type I interferon. *J Exp Med* 187: 79-87
384. Haskard DO, Cavender D, Maliakkal D, Ziff M. 1989. T-cell adhesion to endothelial cells in systemic lupus erythematosis. *Rheumatol Int* 9: 33-7
385. Kamphuis E, Junt T, Waibler Z, Forster R, Kalinke U. 2006. Type I interferons directly regulate lymphocyte recirculation and cause transient blood lymphopenia. *Blood* 108: 3253-61
386. Segerer S, Mac KM, Regele H, Kerjaschki D, Schlondorff D. 1999. Expression of the C-C chemokine receptor 5 in human kidney diseases. *Kidney Int* 56: 52-64
387. Iellem A, Mariani M, Lang R, Recalde H, Panina-Bordignon P, Sinigaglia F, D'Ambrosio D. 2001. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4(+)CD25(+) regulatory T cells. *J Exp Med* 194: 847-53

388. Chan RW, Lai FM, Li EK, Tam LS, Chow KM, Li PK, Szeto CC. 2006. Messenger RNA expression of RANTES in the urinary sediment of patients with lupus nephritis. *Nephrology (Carlton)* 11: 219-25
389. Miyata M, Ito O, Kobayashi H, Sasajima T, Ohira H, Suzuki S, Kasukawa R. 2001. CpG-DNA derived from sera in systemic lupus erythematosus enhances ICAM-1 expression on endothelial cells. *Ann Rheum Dis* 60: 685-9
390. Linker-Israeli M, Bakke AC, Kitridou RC, Gendler S, Gillis S, Horwitz DA. 1983. Defective production of interleukin 1 and interleukin 2 in patients with systemic lupus erythematosus (SLE). *J Immunol* 130: 2651-5
391. Anolik JH, Aringer M. 2005. New treatments for SLE: cell-depleting and anti-cytokine therapies. *Best Pract Res Clin Rheumatol* 19: 859-78
392. Vielhauer V, Anders HJ, Schlondorff D. 2007. Chemokines and chemokine receptors as therapeutic targets in lupus nephritis. *Semin Nephrol* 27: 81-97
393. Liu YJ. 2005. IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23: 275-306
394. Borg FA, Isenberg DA. 2007. Syndromes and complications of interferon therapy. *Curr Opin Rheumatol* 19: 61-6
395. Asselin-Paturel C, Trinchieri G. 2005. Production of type I interferons: plasmacytoid dendritic cells and beyond. *J Exp Med* 202: 461-5
396. Decker P. 2011. Neutrophils and interferon-alpha-producing cells: who produces interferon in lupus? *Arthritis Res Ther* 13: 118
397. Jego G, Palucka AK, Blanck JP, Chalouni C, Pascual V, Banchereau J. 2003. Plasmacytoid dendritic cells induce plasma cell differentiation through type I interferon and interleukin 6. *Immunity* 19: 225-34
398. Merrill JT, Wallace DJ, Petri M, Kirou KA, Yao Y, White WI, Robbie G, Levin R, Berney SM, Chindalore V, Olsen N, Richman L, Le C, Jallal B, White B, Lupus Interferon Skin Activity Study I. 2011. Safety profile and clinical activity of sifalimumab, a fully human anti-interferon alpha monoclonal antibody, in systemic lupus erythematosus: a phase I, multicentre, double-blind randomised study. *Ann Rheum Dis* 70: 1905-13
399. McBride JM, Jiang J, Abbas AR, Morimoto A, Li J, Maciuca R, Townsend M, Wallace DJ, Kennedy WP, Drappa J. 2012. Safety and pharmacodynamics of rontalizumab in patients with systemic lupus erythematosus: results of a phase I, placebo-controlled, double-blind, dose-escalation study. *Arthritis Rheum* 64: 3666-76
400. Lauwerys BR, Hachulla E, Spertini F, Lazaro E, Jorgensen C, Mariette X, Haelterman E, Grouard-Vogel G, Fanget B, Dhellin O, Vandepapeliere P, Houssiau FA. 2013. Down-regulation of interferon signature in systemic lupus erythematosus patients by active immunization with interferon alpha-kinoid. *Arthritis Rheum* 65: 447-56
401. Zhang J, Roschke V, Baker KP, Wang Z, Alarcon GS, Fessler BJ, Bastian H, Kimberly RP, Zhou T. 2001. Cutting edge: a role for B lymphocyte stimulator in systemic lupus erythematosus. *J Immunol* 166: 6-10
402. Kim SS, Kirou KA, Erkan D. 2012. Belimumab in systemic lupus erythematosus: an update for clinicians. *Ther Adv Chronic Dis* 3: 11-23
403. O'Garra A, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C. 2008. Strategies for use of IL-10 or its antagonists in human disease. *Immunol Rev* 223: 114-31
404. Illei GG, Shirota Y, Yarboro CH, Daruwalla J, Tackey E, Takada K, Fleisher T, Balow JE, Lipsky PE. 2010. Tocilizumab in systemic lupus erythematosus: data on safety, preliminary efficacy, and impact on circulating plasma cells from an open-label phase I dosage-escalation study. *Arthritis Rheum* 62: 542-52
405. Llorente L, Richaud-Patin Y, Garcia-Padilla C, Claret E, Jakez-Ocampo J, Cardiel MH, Alcocer-Varela J, Grangeot-Keros L, Alarcon-Segovia D, Wijdenes J, Galanaud P, Emilie D. 2000. Clinical and biologic effects of anti-interleukin-10 monoclonal antibody administration in systemic lupus erythematosus. *Arthritis Rheum* 43: 1790-800

406. Lieberman LA, Tsokos GC. 2010. The IL-2 defect in systemic lupus erythematosus disease has an expansive effect on host immunity. *J Biomed Biotechnol* 2010: 740619
407. Yu SL, Kuan WP, Wong CK, Li EK, Tam LS. 2012. Immunopathological roles of cytokines, chemokines, signaling molecules, and pattern-recognition receptors in systemic lupus erythematosus. *Clin Dev Immunol* 2012: 715190
408. Funauchi M, Sugishima H, Minoda M, Horiuchi A. 1991. Effect of interferon-gamma on B lymphocytes of patients with systemic lupus erythematosus. *J Rheumatol* 18: 368-72
409. Singh RR. 2003. IL-4 and many roads to lupuslike autoimmunity. *Clin Immunol* 108: 73-9
410. Dong W, Zhu P, Wang Y, Wang Z. 2011. Follicular helper T cells in systemic lupus erythematosus: a potential therapeutic target. *Autoimmun Rev* 10: 299-304
411. Shin MS, Lee N, Kang I. 2011. Effector T-cell subsets in systemic lupus erythematosus: update focusing on Th17 cells. *Curr Opin Rheumatol* 23: 444-8
412. Eriksson C, Eneslatt K, Ivanoff J, Rantapaa-Dahlqvist S, Sundqvist KG. 2003. Abnormal expression of chemokine receptors on T-cells from patients with systemic lupus erythematosus. *Lupus* 12: 766-74
413. Lit LC, Wong CK, Tam LS, Li EK, Lam CW. 2006. Raised plasma concentration and ex vivo production of inflammatory chemokines in patients with systemic lupus erythematosus. *Ann Rheum Dis* 65: 209-15
414. Decalf J, Fernandes S, Longman R, Ahloulay M, Audat F, Lefrerre F, Rice CM, Pol S, Albert ML. 2007. Plasmacytoid dendritic cells initiate a complex chemokine and cytokine network and are a viable drug target in chronic HCV patients. *J Exp Med* 204: 2423-37
415. Qian C, An H, Yu Y, Liu S, Cao X. 2007. TLR agonists induce regulatory dendritic cells to recruit Th1 cells via preferential IP-10 secretion and inhibit Th1 proliferation. *Blood* 109: 3308-15
416. Agrawal S, Gupta S. 2011. TLR1/2, TLR7, and TLR9 signals directly activate human peripheral blood naive and memory B cell subsets to produce cytokines, chemokines, and hematopoietic growth factors. *J Clin Immunol* 31: 89-98
417. Zhao X, Tang Y, Qu B, Cui H, Wang S, Wang L, Luo X, Huang X, Li J, Chen S, Shen N. 2010. MicroRNA-125a contributes to elevated inflammatory chemokine RANTES levels via targeting KLF13 in systemic lupus erythematosus. *Arthritis Rheum* 62: 3425-35
418. Cho CS, Cho ML, Chen PP, Min SY, Hwang SY, Park KS, Kim WU, Min DJ, Min JK, Park SH, Kim HY. 2002. Antiphospholipid antibodies induce monocyte chemoattractant protein-1 in endothelial cells. *J Immunol* 168: 4209-15
419. Rovin BH, Lu L, Marsh CB. 2001. Lymphocytes induce monocyte chemoattractant protein-1 production by renal cells after Fc gamma receptor cross-linking: role of IL-1beta. *J Leukoc Biol* 69: 435-9
420. Imaizumi T, Aizawa T, Hayakari R, Xing F, Meng P, Tsuruga K, Matsumiya T, Yoshida H, Wang L, Tatsuta T, Tanaka H. 2014. Tumor necrosis factor-alpha synergistically enhances polyinosinic-polycytidylic acid-induced toll-like receptor 3 signaling in cultured normal human mesangial cells: possible involvement in the pathogenesis of lupus nephritis. *Clin Exp Nephrol*
421. Chambers SA, Isenberg D. 2005. Anti-B cell therapy (rituximab) in the treatment of autoimmune diseases. *Lupus* 14: 210-4
422. Silverman GJ, Weisman S. 2003. Rituximab therapy and autoimmune disorders: prospects for anti-B cell therapy. *Arthritis Rheum* 48: 1484-92
423. Barun B, Bar-Or A. 2012. Treatment of multiple sclerosis with anti-CD20 antibodies. *Clin Immunol* 142: 31-7
424. Leandro MJ, Edwards JC, Cambridge G, Ehrenstein MR, Isenberg DA. 2002. An open study of B lymphocyte depletion in systemic lupus erythematosus. *Arthritis Rheum* 46: 2673-7

425. Lu TY, Ng KP, Cambridge G, Leandro MJ, Edwards JC, Ehrenstein M, Isenberg DA. 2009. A retrospective seven-year analysis of the use of B cell depletion therapy in systemic lupus erythematosus at University College London Hospital: the first fifty patients. *Arthritis Rheum* 61: 482-7
426. Looney RJ, Anolik JH, Campbell D, Felgar RE, Young F, Arend LJ, Sloand JA, Rosenblatt J, Sanz I. 2004. B cell depletion as a novel treatment for systemic lupus erythematosus: a phase I/II dose-escalation trial of rituximab. *Arthritis Rheum* 50: 2580-9
427. Jonsdottir T, Gunnarsson I, Risselada A, Henriksson EW, Klareskog L, van Vollenhoven RF. 2008. Treatment of refractory SLE with rituximab plus cyclophosphamide: clinical effects, serological changes, and predictors of response. *Ann Rheum Dis* 67: 330-4
428. Catapano F, Chaudhry AN, Jones RB, Smith KG, Jayne DW. 2010. Long-term efficacy and safety of rituximab in refractory and relapsing systemic lupus erythematosus. *Nephrol Dial Transplant* 25: 3586-92
429. Vital EM, Dass S, Buch MH, Henshaw K, Pease CT, Martin MF, Ponchel F, Rawstron AC, Emery P. 2011. B cell biomarkers of rituximab responses in systemic lupus erythematosus. *Arthritis Rheum* 63: 3038-47
430. Terrier B, Amoura Z, Ravaud P, Hachulla E, Jouenne R, Combe B, Bonnet C, Cacoub P, Cantagrel A, de Bandt M, Fain O, Fautrel B, Gaudin P, Godeau B, Harle JR, Hot A, Kahn JE, Lambotte O, Larroche C, Leone J, Meyer O, Pallot-Prades B, Pertuiset E, Quartier P, Schaerverbeke T, Sibilia J, Somogyi A, Soubrier M, Vignon E, Bader-Meunier B, Mariette X, Gottenberg JE, Club Rhumatismes et I. 2010. Safety and efficacy of rituximab in systemic lupus erythematosus: results from 136 patients from the French AutoImmunity and Rituximab registry. *Arthritis Rheum* 62: 2458-66
431. Merrill JT, Neuwelt CM, Wallace DJ, Shanahan JC, Latinis KM, Oates JC, Utset TO, Gordon C, Isenberg DA, Hsieh HJ, Zhang D, Brunetta PG. 2010. Efficacy and safety of rituximab in moderately-to-severely active systemic lupus erythematosus: the randomized, double-blind, phase II/III systemic lupus erythematosus evaluation of rituximab trial. *Arthritis Rheum* 62: 222-33
432. Rovin BH, Furie R, Latinis K, Looney RJ, Fervenza FC, Sanchez-Guerrero J, Maciuca R, Zhang D, Garg JP, Brunetta P, Appel G, Group LI. 2012. Efficacy and safety of rituximab in patients with active proliferative lupus nephritis: the Lupus Nephritis Assessment with Rituximab study. *Arthritis Rheum* 64: 1215-26
433. Villarroel MC, Hidalgo M, Jimeno A. 2009. Mycophenolate mofetil: An update. *Drugs Today (Barc)* 45: 521-32
434. Dall'Era M. 2011. Mycophenolate mofetil in the treatment of systemic lupus erythematosus. *Curr Opin Rheumatol* 23: 454-8
435. Cambridge G, Leandro MJ, Teodorescu M, Manson J, Rahman A, Isenberg DA, Edwards JC. 2006. B cell depletion therapy in systemic lupus erythematosus: effect on autoantibody and antimicrobial antibody profiles. *Arthritis Rheum* 54: 3612-22
436. Ng KP, Cambridge G, Leandro MJ, Edwards JC, Ehrenstein M, Isenberg DA. 2007. B cell depletion therapy in systemic lupus erythematosus: long-term follow-up and predictors of response. *Ann Rheum Dis* 66: 1259-62
437. Cambridge G, Isenberg DA, Edwards JC, Leandro MJ, Migone TS, Teodorescu M, Stohl W. 2008. B cell depletion therapy in systemic lupus erythematosus: relationships among serum B lymphocyte stimulator levels, autoantibody profile and clinical response. *Ann Rheum Dis* 67: 1011-6
438. Anolik JH, Barnard J, Owen T, Zheng B, Kemshetti S, Looney RJ, Sanz I. 2007. Delayed memory B cell recovery in peripheral blood and lymphoid tissue in systemic lupus erythematosus after B cell depletion therapy. *Arthritis Rheum* 56: 3044-56
439. Tamimoto Y, Horiuchi T, Tsukamoto H, Otsuka J, Mitoma H, Kimoto Y, Nakashima H, Muta K, Abe Y, Kiyohara C, Ueda A, Nagasawa K, Yoshizawa S, Shimoda T, Harada M. 2008. A dose-escalation study of rituximab for treatment of systemic

lupus erythematosus and Evans' syndrome: immunological analysis of B cells, T cells and cytokines. *Rheumatology (Oxford)* 47: 821-7
440. Sfikakis PP, Souliotis VL, Fragiadaki KG, Moutsopoulos HM, Boletis JN, Theofilopoulos AN. 2007. Increased expression of the FoxP3 functional marker of regulatory T cells following B cell depletion with rituximab in patients with lupus nephritis. *Clin Immunol* 123: 66-73
441. Gunnarsson I, Sundelin B, Jonsdottir T, Jacobson SH, Henriksson EW, van Vollenhoven RF. 2007. Histopathologic and clinical outcome of rituximab treatment in patients with cyclophosphamide-resistant proliferative lupus nephritis. *Arthritis Rheum* 56: 1263-72
442. Piccio L, Naismith RT, Trinkaus K, Klein RS, Parks BJ, Lyons JA, Cross AH. 2010. Changes in B- and T-lymphocyte and chemokine levels with rituximab treatment in multiple sclerosis. *Arch Neurol* 67: 707-14
443. Tveita AA. 2010. The danger model in deciphering autoimmunity. *Rheumatology (Oxford)* 49: 632-9
444. Pathak S, Mohan C. 2011. Cellular and molecular pathogenesis of systemic lupus erythematosus: lessons from animal models. *Arthritis Res Ther* 13: 241
445. Spies JM, Westland KW, Bonner JG, Pollard JD. 1995. Intraneural activated T cells cause focal breakdown of the blood-nerve barrier. *Brain* 118 (Pt 4): 857-68
446. Pollard JD, Westland KW, Harvey GK, Jung S, Bonner J, Spies JM, Toyka KV, Hartung HP. 1995. Activated T cells of nonneural specificity open the blood-nerve barrier to circulating antibody. *Ann Neurol* 37: 467-75
447. Westland KW, Pollard JD, Sander S, Bonner JG, Linington C, McLeod JG. 1999. Activated non-neural specific T cells open the blood-brain barrier to circulating antibodies. *Brain* 122 (Pt 7): 1283-91
448. Rudick RA, Trapp BD. 2009. Gray-matter injury in multiple sclerosis. *N Engl J Med* 361: 1505-6
449. Diamond B, Volpe BT. 2012. A model for lupus brain disease. *Immunol Rev* 248: 56-67
450. Liu K, Mohan C. 2006. What do mouse models teach us about human SLE? *Clin Immunol* 119: 123-30
451. Hay EM, Bacon PA, Gordon C, Isenberg DA, Maddison P, Snaith ML, Symmons DP, Viner N, Zoma A. 1993. The BILAG index: a reliable and valid instrument for measuring clinical disease activity in systemic lupus erythematosus. *Q J Med* 86: 447-58
452. Liang MH, Socher SA, Larson MG, Schur PH. 1989. Reliability and validity of six systems for the clinical assessment of disease activity in systemic lupus erythematosus. *Arthritis Rheum* 32: 1107-18
453. Yee CS, Isenberg DA, Prabu A, Sokoll K, Teh LS, Rahman A, Bruce IN, Griffiths B, Akil M, McHugh N, D'Cruz D, Khamashta MA, Maddison P, Zoma A, Gordon C. 2008. BILAG-2004 index captures systemic lupus erythematosus disease activity better than SLEDAI-2000. *Ann Rheum Dis* 67: 873-6
454. Walz LeBlanc BA, Gladman DD, Urowitz MB. 1994. Serologically active clinically quiescent systemic lupus erythematosus--predictors of clinical flares. *J Rheumatol* 21: 2239-41
455. Winkler IG, Pettit AR, Raggatt LJ, Jacobsen RN, Forristal CE, Barbier V, Nowlan B, Cisterne A, Bendall LJ, Sims NA, Levesque JP. 2012. Hematopoietic stem cell mobilizing agents G-CSF, cyclophosphamide or AMD3100 have distinct mechanisms of action on bone marrow HSC niches and bone formation. *Leukemia* 26: 1594-601
456. Winkler IG, Bendall LJ, Forristal CE, Helwani F, Nowlan B, Barbier V, Shen Y, Cisterne A, Sedger LM, Levesque JP. 2013. B-lymphopoiesis is stopped by mobilizing doses of G-CSF and is rescued by overexpression of the anti-apoptotic protein Bcl2. *Haematologica* 98: 325-33

457. Li EK, Tam LS, Zhu TY, Li M, Kwok CL, Li TK, Leung YY, Wong KC, Szeto CC. 2009. Is combination rituximab with cyclophosphamide better than rituximab alone in the treatment of lupus nephritis? *Rheumatology (Oxford)* 48: 892-8
458. Thewissen M, Somers V, Hellings N, Fraussen J, Damoiseaux J, Stinissen P. 2007. CD4+CD28null T cells in autoimmune disease: pathogenic features and decreased susceptibility to immunoregulation. *J Immunol* 179: 6514-23
459. Tortorella C, Schulze-Koops H, Thomas R, Splawski JB, Davis LS, Picker LJ, Lipsky PE. 1995. Expression of CD45RB and CD27 identifies subsets of CD4+ memory T cells with different capacities to induce B cell differentiation. *J Immunol* 155: 149-62
460. Amoroso K, Lipsky PE. 1990. Frequency of human B cells that differentiate in response to anti-CD3-activated T cells. *J Immunol* 145: 3155-61
461. Leandro MJ. 2013. B-cell subpopulations in humans and their differential susceptibility to depletion with anti-CD20 monoclonal antibodies. *Arthritis Res Ther* 15 Suppl 1: S3
462. Libri V, Azevedo RI, Jackson SE, Di Mitri D, Lachmann R, Fuhrmann S, Vukmanovic-Stejic M, Yong K, Battistini L, Kern F, Soares MV, Akbar AN. 2011. Cytomegalovirus infection induces the accumulation of short-lived, multifunctional CD4+CD45RA+CD27+ T cells: the potential involvement of interleukin-7 in this process. *Immunology* 132: 326-39
463. Choudhury A, Cohen PL, Eisenberg RA. 2010. B cells require "nurturing" by CD4 T cells during development in order to respond in chronic graft-versus-host model of systemic lupus erythematosus. *Clin Immunol* 136: 105-15
464. Zhou Z, Niu H, Zheng YY, Morel L. 2011. Autoreactive marginal zone B cells enter the follicles and interact with CD4+ T cells in lupus-prone mice. *BMC Immunol* 12: 7
465. Yoshimoto K, Takahashi Y, Ogasawara M, Setoyama Y, Suzuki K, Tsuzaka K, Abe T, Takeuchi T. 2006. Aberrant expression of BAFF in T cells of systemic lupus erythematosus, which is recapitulated by a human T cell line, Loucy. *Int Immunol* 18: 1189-96
466. Harigai M, Kawamoto M, Hara M, Kubota T, Kamatani N, Miyasaka N. 2008. Excessive production of IFN-gamma in patients with systemic lupus erythematosus and its contribution to induction of B lymphocyte stimulator/B cell-activating factor/TNF ligand superfamily-13B. *J Immunol* 181: 2211-9
467. Thien M, Phan TG, Gardam S, Amesbury M, Basten A, Mackay F, Brink R. 2004. Excess BAFF rescues self-reactive B cells from peripheral deletion and allows them to enter forbidden follicular and marginal zone niches. *Immunity* 20: 785-98
468. Lesley R, Xu Y, Kalled SL, Hess DM, Schwab SR, Shu HB, Cyster JG. 2004. Reduced competitiveness of autoantigen-engaged B cells due to increased dependence on BAFF. *Immunity* 20: 441-53
469. Jacobi AM, Huang W, Wang T, Freimuth W, Sanz I, Furie R, Mackay M, Aranow C, Diamond B, Davidson A. 2010. Effect of long-term belimumab treatment on B cells in systemic lupus erythematosus: extension of a phase II, double-blind, placebo-controlled, dose-ranging study. *Arthritis Rheum* 62: 201-10
470. Takeno M, Nagafuchi H, Kaneko S, Wakisaka S, Oneda K, Takeba Y, Yamashita N, Suzuki N, Kaneoka H, Sakane T. 1997. Autoreactive T cell clones from patients with systemic lupus erythematosus support polyclonal autoantibody production. *J Immunol* 158: 3529-38
471. Shirota Y, Yarboro C, Fischer R, Pham TH, Lipsky P, Illei GG. 2013. Impact of anti-interleukin-6 receptor blockade on circulating T and B cell subsets in patients with systemic lupus erythematosus. *Ann Rheum Dis* 72: 118-28
472. Kitani A, Hara M, Hirose T, Norioka K, Harigai M, Hirose W, Suzuki K, Kawakami M, Kawagoe M, Nakamura H. 1989. Heterogeneity of B cell responsiveness to interleukin 4, interleukin 6 and low molecular weight B cell growth factor in discrete stages of B cell activation in patients with systemic lupus erythematosus. *Clin Exp Immunol* 77: 31-6

473. Sudo T, Ito M, Ogawa Y, Iizuka M, Kodama H, Kunisada T, Hayashi S, Ogawa M, Sakai K, Nishikawa S. 1989. Interleukin 7 production and function in stromal cell-dependent B cell development. *J Exp Med* 170: 333-8
474. Colonna-Romano G, Bulati M, Aquino A, Pellicano M, Vitello S, Lio D, Candore G, Caruso C. 2009. A double-negative (IgD-CD27-) B cell population is increased in the peripheral blood of elderly people. *Mech Ageing Dev* 130: 681-90
475. Kovaiou RD, Weiskirchner I, Keller M, Pfister G, Cioca DP, Grubeck-Loebenstein B. 2005. Age-related differences in phenotype and function of CD4+ T cells are due to a phenotypic shift from naive to memory effector CD4+ T cells. *Int Immunol* 17: 1359-66
476. Kuhnert M, Strohmeier R, Stegmuller M, Halberstadt E. 1998. Changes in lymphocyte subsets during normal pregnancy. *Eur J Obstet Gynecol Reprod Biol* 76: 147-51
477. Watanabe M, Iwatani Y, Kaneda T, Hidaka Y, Mitsuda N, Morimoto Y, Amino N. 1997. Changes in T, B, and NK lymphocyte subsets during and after normal pregnancy. *Am J Reprod Immunol* 37: 368-77
478. Mahmoud F, Abul H, Omu A, Al-Rayes S, Haines D, Whaley K. 2001. Pregnancy-associated changes in peripheral blood lymphocyte subpopulations in normal Kuwaiti women. *Gynecol Obstet Invest* 52: 232-6
479. Medina KL, Kincade PW. 1994. Pregnancy-related steroids are potential negative regulators of B lymphopoiesis. *Proc Natl Acad Sci U S A* 91: 5382-6
480. Arck PC, Hecher K, Solano ME. 2014. B Cells in Pregnancy: Functional Promiscuity or Tailored Function? *Biol Reprod*
481. Pope RM. 1990. Immunoregulatory mechanisms present in the maternal circulation during pregnancy. *Baillieres Clin Rheumatol* 4: 33-52
482. Muzzio D, Zenclussen AC, Jensen F. 2013. The role of B cells in pregnancy: the good and the bad. *Am J Reprod Immunol* 69: 408-12
483. Muzzio D, Zygmunt M, Jensen F. 2014. The role of pregnancy-associated hormones in the development and function of regulatory B cells. *Front Endocrinol (Lausanne)* 5: 39
484. Venkatesh J, Peeva E, Xu X, Diamond B. 2006. Cutting Edge: Hormonal milieu, not antigenic specificity, determines the mature phenotype of autoreactive B cells. *J Immunol* 176: 3311-4
485. Gutierrez MA, Molina JF, Jara LJ, Garcia C, Gutierrez-Urena S, Cuellar ML, Gharavi A, Espinoza LR. 1996. Prolactin-induced immunoglobulin and autoantibody production by peripheral blood mononuclear cells from systemic lupus erythematosus and normal individuals. *Int Arch Allergy Immunol* 109: 229-35
486. Kanda N, Tsuchida T, Tamaki K. 1999. Estrogen enhancement of anti-double-stranded DNA antibody and immunoglobulin G production in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Arthritis Rheum* 42: 328-37
487. Jensen F, Wallukat G, Herse F, Budner O, El-Mousleh T, Costa SD, Dechend R, Zenclussen AC. 2012. CD19+CD5+ cells as indicators of preeclampsia. *Hypertension* 59: 861-8
488. Tanaka Y, Saito K, Shirakawa F, Ota T, Suzuki H, Eto S, Yamashita U. 1988. Production of B cell-stimulating factors by B cells in patients with systemic lupus erythematosus. *J Immunol* 141: 3043-9
489. Abu-Rish EY, Amrani Y, Browning MJ. 2013. Toll-like receptor 9 activation induces expression of membrane-bound B-cell activating factor (BAFF) on human B cells and leads to increased proliferation in response to both soluble and membrane-bound BAFF. *Rheumatology (Oxford)* 52: 1190-201
490. Carlsten H, Nilsson N, Jonsson R, Tarkowski A. 1991. Differential effects of oestrogen in murine lupus: acceleration of glomerulonephritis and amelioration of T cell-mediated lesions. *J Autoimmun* 4: 845-56

491. Clowse ME, Magder LS, Petri M. 2011. The clinical utility of measuring complement and anti-dsDNA antibodies during pregnancy in patients with systemic lupus erythematosus. *J Rheumatol* 38: 1012-6
492. Kuhn A, Sticherling M, Bonsmann G. 2007. Clinical manifestations of cutaneous lupus erythematosus. *J Dtsch Dermatol Ges* 5: 1124-37
493. David-Bajar KM, Davis BM. 1997. Pathology, immunopathology, and immunohistochemistry in cutaneous lupus erythematosus. *Lupus* 6: 145-57
494. Tang X, Huang Y, Deng W, Tang L, Weng W, Zhang X. 2010. Clinical and serologic correlations and autoantibody clusters in systemic lupus erythematosus: a retrospective review of 917 patients in South China. *Medicine (Baltimore)* 89: 62-7
495. Thompson D, Juby A, Davis P. 1993. The clinical significance of autoantibody profiles in patients with systemic lupus erythematosus. *Lupus* 2: 15-9
496. Okon LG, Werth VP. 2013. Cutaneous lupus erythematosus: diagnosis and treatment. *Best Pract Res Clin Rheumatol* 27: 391-404
497. Zeng FQ, Yin RF, Tan GZ, Guo Q, Xu DQ. 2004. Characterization of DNA antigens from immune complexes deposited in the skin of patients with systemic lupus erythematosus. *Chin Med J (Engl)* 117: 1066-71
498. Steinmetz OM, Velden J, Kneissler U, Marx M, Klein A, Helmchen U, Stahl RA, Panzer U. 2008. Analysis and classification of B-cell infiltrates in lupus and ANCA-associated nephritis. *Kidney Int* 74: 448-57
499. Marinkovic T, Garin A, Yokota Y, Fu YX, Ruddle NH, Furtado GC, Lira SA. 2006. Interaction of mature CD3+CD4+ T cells with dendritic cells triggers the development of tertiary lymphoid structures in the thyroid. *J Clin Invest* 116: 2622-32
500. Takemura S, Klimiuk PA, Braun A, Goronzy JJ, Weyand CM. 2001. T cell activation in rheumatoid synovium is B cell dependent. *J Immunol* 167: 4710-8
501. Iwata S, Saito K, Tokunaga M, Yamaoka K, Nawata M, Yukawa S, Hanami K, Fukuyo S, Miyagawa I, Kubo S, Tanaka Y. 2011. Phenotypic changes of lymphocytes in patients with systemic lupus erythematosus who are in longterm remission after B cell depletion therapy with rituximab. *J Rheumatol* 38: 633-41
502. Spronk PE, Horst G, Van Der Gun BT, Limburg PC, Kallenberg CG. 1996. Anti-dsDNA production coincides with concurrent B and T cell activation during development of active disease in systemic lupus erythematosus (SLE). *Clin Exp Immunol* 104: 446-53
503. Takaya M, Ichikawa Y, Shimizu H, Uchiyama M, Moriuchi J, Arimori S. 1990. Expression of MHC class II antigens and other T cell activation antigens on T cells and salivary duct epithelial cells in the salivary gland of cases of Sjogren's syndrome. *Tokai J Exp Clin Med* 15: 27-33
504. Groen H, Aslander M, Bootsma H, van der Mark TW, Kallenberg CG, Postma DS. 1993. Bronchoalveolar lavage cell analysis and lung function impairment in patients with systemic lupus erythematosus (SLE). *Clin Exp Immunol* 94: 127-33
505. Giacomelli R, Matucci-Cerinic M, Cipriani P, Ghersetich I, Lattanzio R, Pavan A, Pignone A, Cagnoni ML, Lotti T, Tonietti G. 1998. Circulating Vdelta1+ T cells are activated and accumulate in the skin of systemic sclerosis patients. *Arthritis Rheum* 41: 327-34
506. Undale AH, van den Elsen PJ, Celis E. 2004. Antigen-independent acquisition of MHC class II molecules by human T lymphocytes. *Int Immunol* 16: 1523-33
507. Pugh-Bernard AE, Cambier JC. 2006. B cell receptor signaling in human systemic lupus erythematosus. *Curr Opin Rheumatol* 18: 451-5
508. Jenks SA, Sanz I. 2009. Altered B cell receptor signaling in human systemic lupus erythematosus. *Autoimmun Rev* 8: 209-13
509. Nakano S, Morimoto S, Suzuki J, Nozawa K, Amano H, Tokano Y, Takasaki Y. 2008. Role of pathogenic auto-antibody production by Toll-like receptor 9 of B cells in active systemic lupus erythematosus. *Rheumatology (Oxford)* 47: 145-9
510. Bierer BE, Hahn WC. 1993. T cell adhesion, avidity regulation and signaling: a molecular analysis of CD2. *Semin Immunol* 5: 249-61

511. Kranzer K, Bauer M, Lipford GB, Heeg K, Wagner H, Lang R. 2000. CpG-oligodeoxynucleotides enhance T-cell receptor-triggered interferon-gamma production and up-regulation of CD69 via induction of antigen-presenting cell-derived interferon type I and interleukin-12. *Immunology* 99: 170-8
512. Rivero SJ, Diaz-Jouanen E, Alarcon-Segovia D. 1978. Lymphopenia in systemic lupus erythematosus. Clinical, diagnostic, and prognostic significance. *Arthritis Rheum* 21: 295-305
513. Mirzayan MJ, Schmidt RE, Witte T. 2000. Prognostic parameters for flare in systemic lupus erythematosus. *Rheumatology (Oxford)* 39: 1316-9
514. Wenzel J, Bauer R, Uerlich M, Bieber T, Boehm I. 2002. The value of lymphocytopenia as a marker of systemic involvement in cutaneous lupus erythematosus. *Br J Dermatol* 146: 869-71
515. Mittal KK, Rossen RD, Sharp JT, Lidsky MD, Butler WT. 1970. Lymphocyte cytotoxic antibodies in systemic lupus erythematosus. *Nature* 225: 1255-6
516. Stafford HA, Chen AE, Anderson CJ, Paul AG, Wyatt EL, Lee LA, Neas BR. 1997. Anti-ribosomal and 'P-peptide'-specific autoantibodies bind to T lymphocytes. *Clin Exp Immunol* 109: 12-9
517. Winfield JB, Mimura T, Fernsten PD. 1992. Autoantibodies to CD45 in systemic lupus erythematosus. *Int J Clin Lab Res* 21: 292-5
518. Garcia-Valladares I, Atisha-Fregoso Y, Richaud-Patin Y, Jakez-Ocampo J, Soto-Vega E, Elias-Lopez D, Carrillo-Maravilla E, Cabiedes J, Ruiz-Arguelles A, Llorente L. 2006. Diminished expression of complement regulatory proteins (CD55 and CD59) in lymphocytes from systemic lupus erythematosus patients with lymphopenia. *Lupus* 15: 600-5
519. Dhir V, Singh AP, Aggarwal A, Naik S, Misra R. 2009. Increased T-lymphocyte apoptosis in lupus correlates with disease activity and may be responsible for reduced T-cell frequency: a cross-sectional and longitudinal study. *Lupus* 18: 785-91
520. Williams RC, Jr., Bankhurst AD. 1978. Clinical immunologic studies in systemic lupus erythematosus. *Arthritis Rheum* 21: S202-9
521. Norman MU, Hickey MJ. 2005. Mechanisms of lymphocyte migration in autoimmune disease. *Tissue Antigens* 66: 163-72
522. Caligaris-Cappio F, Bergui L, Tesio L, Ziano R, Camussi G. 1985. HLA-Dr+ T cells of the Leu 3 (helper) type infiltrate the kidneys of patients with systemic lupus erythematosus. *Clin Exp Immunol* 59: 185-9
523. Contin-Bordes C, Lazaro E, Richez C, Jacquemin C, Caubet O, Douchet I, Viallard JF, Moreau JF, Pellegrin JL, Blanco P. 2011. Expansion of myelin autoreactive CD8+ T lymphocytes in patients with neuropsychiatric systemic lupus erythematosus. *Ann Rheum Dis* 70: 868-71
524. Stummvoll GH, Aringer M, Grisar J, Steiner CW, Smolen JS, Knobler R, Graninger WB. 2004. Increased transendothelial migration of scleroderma lymphocytes. *Ann Rheum Dis* 63: 569-74
525. Kaneko H, Saito K, Hashimoto H, Yagita H, Okumura K, Azuma M. 1996. Preferential elimination of CD28+ T cells in systemic lupus erythematosus (SLE) and the relation with activation-induced apoptosis. *Clin Exp Immunol* 106: 218-29
526. Odum N, Yoshizumi H, Okamoto Y, Kamikawaji N, Kimura A, Nishimura Y, Sasazuki T. 1992. Signal transduction by HLA class II molecules in human T cells: induction of LFA-1-dependent and independent adhesion. *Hum Immunol* 35: 71-84
527. Nielsen M, Gerwien J, Geisler C, Ropke C, Svejgaard A, Odum N. 1998. MHC class II ligation induces CD58 (LFA-3)-mediated adhesion in human T cells. *Exp Clin Immunogenet* 15: 61-8
528. Pober JS, Collins T, Gimbrone MA, Jr., Cotran RS, Gitlin JD, Fiers W, Clayberger C, Krensky AM, Burakoff SJ, Reiss CS. 1983. Lymphocytes recognize human vascular endothelial and dermal fibroblast Ia antigens induced by recombinant immune interferon. *Nature* 305: 726-9

529. Savage CO, Brooks CJ, Harcourt GC, Picard JK, King W, Sansom DM, Willcox N. 1995. Human vascular endothelial cells process and present autoantigen to human T cell lines. *Int Immunol* 7: 471-9
530. Rhodes LE, Joyce M, West DC, Strickland I, Friedmann PS. 1996. Comparison of changes in endothelial adhesion molecule expression following UVB irradiation of skin and a human dermal microvascular cell line (HMEC-1). *Photodermatol Photoimmunol Photomed* 12: 114-21
531. McMurray RW. 1996. Adhesion molecules in autoimmune disease. *Semin Arthritis Rheum* 25: 215-33
532. Reefman E, Kuiper H, Limburg PC, Kallenberg CG, Bijl M. 2008. Type I interferons are involved in the development of ultraviolet B-induced inflammatory skin lesions in systemic lupus erythaematosus patients. *Ann Rheum Dis* 67: 11-8
533. Aksu K, Donmez A, Keser G. 2012. Inflammation-induced thrombosis: mechanisms, disease associations and management. *Curr Pharm Des* 18: 1478-93
534. Daca A, Czuszynska Z, Smolenska Z, Zdrojewski Z, Witkowski JM, Bryl E. 2011. Two systemic lupus erythematosus (SLE) global disease activity indexes--the SLE Disease Activity Index and the Systemic Lupus Activity Measure--demonstrate different correlations with activation of peripheral blood CD4+ T cells. *Hum Immunol* 72: 1160-7
535. Barten LJ, Allington DR, Procacci KA, Rivey MP. 2010. New approaches in the management of multiple sclerosis. *Drug Des Devel Ther* 4: 343-66
536. Carson KR, Focosi D, Major EO, Petrini M, Richey EA, West DP, Bennett CL. 2009. Monoclonal antibody-associated progressive multifocal leucoencephalopathy in patients treated with rituximab, natalizumab, and efalizumab: a Review from the Research on Adverse Drug Events and Reports (RADAR) Project. *Lancet Oncol* 10: 816-24
537. Aksoy S, Harputluoglu H, Kilickap S, Dede DS, Dizdar O, Altundag K, Barista I. 2007. Rituximab-related viral infections in lymphoma patients. *Leuk Lymphoma* 48: 1307-12
538. Sagnelli E, Pisaturo M, Sagnelli C, Coppola N. 2012. Rituximab-based treatment, HCV replication, and hepatic flares. *Clin Dev Immunol* 2012: 945950
539. Hirai Y, Ainoda Y, Shoji T, Fujita T, Yoshinaga K, Shiseki M, Mori N, Teramura M, Totsuka K, Motoji T. 2011. Disseminated cryptococcosis in a non-Hodgkin's lymphoma patient with late-onset neutropenia following rituximab-CHOP chemotherapy: a case report and literature review. *Mycopathologia* 172: 227-32
540. Ettahar N, Legout L, Ajana F, Patoz P, Massongo M, Rose C, Senneville E. 2013. [Cryptococcal osteomyelitis in a patient with a lymphocytic leukemia treated with fludarabine-cyclophosphamide-rituximab]. *J Mycol Med* 23: 57-63
541. Wingfield T, Jani M, Krutikov M, Mayer J, Uriel A, Marks J, Ustianowski AP. 2011. Cryptococcal meningitis in an HIV-negative patient with rheumatoid arthritis treated with rituximab. *Rheumatology (Oxford)* 50: 1725-7
542. Portales-Perez D, Gonzalez-Amaro R, Abud-Mendoza C, Sanchez-Armass S. 1997. Abnormalities in CD69 expression, cytosolic pH and Ca2+ during activation of lymphocytes from patients with systemic lupus erythematosus. *Lupus* 6: 48-56
543. Martin P, Gomez M, Lamana A, Cruz-Adalia A, Ramirez-Huesca M, Ursa MA, Yanez-Mo M, Sanchez-Madrid F. 2010. CD69 association with Jak3/Stat5 proteins regulates Th17 cell differentiation. *Mol Cell Biol* 30: 4877-89
544. Radulovic K, Manta C, Rossini V, Holzmann K, Kestler HA, Wegenka UM, Nakayama T, Niess JH. 2012. CD69 regulates type I IFN-induced tolerogenic signals to mucosal CD4 T cells that attenuate their colitogenic potential. *J Immunol* 188: 2001-13
545. Shinoda K, Tokoyoda K, Hanazawa A, Hayashizaki K, Zehentmeier S, Hosokawa H, Iwamura C, Koseki H, Tumes DJ, Radbruch A, Nakayama T. 2012. Type II membrane protein CD69 regulates the formation of resting T-helper memory. *Proc Natl Acad Sci U S A* 109: 7409-14

546. Feng C, Woodside KJ, Vance BA, El-Khoury D, Canelles M, Lee J, Gress R, Fowlkes BJ, Shores EW, Love PE. 2002. A potential role for CD69 in thymocyte emigration. *Int Immunol* 14: 535-44
547. Hoyer BF, Manz RA, Radbruch A, Hiepe F. 2005. Long-lived plasma cells and their contribution to autoimmunity. *Ann N Y Acad Sci* 1050: 124-33
548. Hoyer BF, Moser K, Hauser AE, Peddinghaus A, Voigt C, Eilat D, Radbruch A, Hiepe F, Manz RA. 2004. Short-lived plasmablasts and long-lived plasma cells contribute to chronic humoral autoimmunity in NZB/W mice. *J Exp Med* 199: 1577-84
549. Mahevas M, Patin P, Huetz F, Descatoire M, Cagnard N, Bole-Feysot C, Le Gallou S, Khellaf M, Fain O, Boutboul D, Galicier L, Ebbo M, Lambotte O, Hamidou M, Bierling P, Godeau B, Michel M, Weill JC, Reynaud CA. 2013. B cell depletion in immune thrombocytopenia reveals splenic long-lived plasma cells. *J Clin Invest* 123: 432-42
550. Starke C, Frey S, Wellmann U, Urbonaviciute V, Herrmann M, Amann K, Schett G, Winkler T, Voll RE. 2011. High frequency of autoantibody-secreting cells and long-lived plasma cells within inflamed kidneys of NZB/W F1 lupus mice. *Eur J Immunol* 41: 2107-12
551. Fragoso-Loyo H, Richaud-Patin Y, Orozco-Narvaez A, Davila-Maldonado L, Atisha-Fregoso Y, Llorente L, Sanchez-Guerrero J. 2007. Interleukin-6 and chemokines in the neuropsychiatric manifestations of systemic lupus erythematosus. *Arthritis Rheum* 56: 1242-50
552. Kulkarni O, Anders HJ. 2008. Chemokines in lupus nephritis. *Front Biosci* 13: 3312-20
553. Kong KO, Tan AW, Thong BY, Lian TY, Cheng YK, Teh CL, Koh ET, Chng HH, Law WG, Lau TC, Leong KP, Leung BP, Howe HS. 2009. Enhanced expression of interferon-inducible protein-10 correlates with disease activity and clinical manifestations in systemic lupus erythematosus. *Clin Exp Immunol* 156: 134-40
554. Tucci M, Ciavarella S, Strippoli S, Dammacco F, Silvestris F. 2009. Oversecretion of cytokines and chemokines in lupus nephritis is regulated by intraparenchymal dendritic cells: a review. *Ann N Y Acad Sci* 1173: 449-57
555. Shirakawa F, Yamashita U, Suzuki H. 1987. Monocyte (macrophage)-specific antibodies in patients with systemic lupus erythematosus (SLE). *J Clin Immunol* 7: 121-9
556. Bai Y, Liu R, Huang D, La Cava A, Tang YY, Iwakura Y, Campagnolo DI, Vollmer TL, Ransohoff RM, Shi FD. 2008. CCL2 recruitment of IL-6-producing CD11b+ monocytes to the draining lymph nodes during the initiation of Th17-dependent B cell-mediated autoimmunity. *Eur J Immunol* 38: 1877-88
557. Berggren O, Hagberg N, Weber G, Alm GV, Ronnblom L, Eloranta ML. 2012. B lymphocytes enhance interferon-alpha production by plasmacytoid dendritic cells. *Arthritis Rheum* 64: 3409-19
558. Morva A, Lemoine S, Achour A, Pers JO, Youinou P, Jamin C. 2012. Maturation and function of human dendritic cells are regulated by B lymphocytes. *Blood* 119: 106-14
559. Li Y, Tucci M, Narain S, Barnes EV, Sobel ES, Segal MS, Richards HB. 2006. Urinary biomarkers in lupus nephritis. *Autoimmun Rev* 5: 383-8
560. Groom JR, Luster AD. 2011. CXCR3 ligands: redundant, collaborative and antagonistic functions. *Immunol Cell Biol* 89: 207-15
561. Sato W, Aranami T, Yamamura T. 2007. Cutting edge: Human Th17 cells are identified as bearing CCR2+CCR5- phenotype. *J Immunol* 178: 7525-9
562. Lewis JE, Fu SM, Gaskin F. 2013. Autoimmunity, end organ damage, and the origin of autoantibodies and autoreactive T cells in systemic lupus erythematosus. *Discov Med* 15: 85-92
563. Ferraccioli G, Tolusso B. 2007. Infections, B cell receptor activation and autoimmunity: different check-point impairments lead to autoimmunity, clonal B cell expansion and fibrosis in different immunological settings. *Autoimmun Rev* 7: 109-13

564. Navratil JS, Ahearn JM. 2001. Apoptosis, clearance mechanisms, and the development of systemic lupus erythematosus. *Curr Rheumatol Rep* 3: 191-8

Appendix 1

BILAG (British Isles Lupus Assessment Group index)

Patient_____ Date_____

All features must be attributable to SLE and refer the last four weeks compared with the prior visit's disease activity.
Indicate and score which features are present: 0 = Not Present, 1 = Improving, 2 = Same, 3 = Worse, 4 = New or Recurrence.

General – MUST BE SLE RELATED		**Neurological - MUST BE SLE RELATED**	
1. Pyrexia (documented)	0 1 2 3 4	24. Deteriorating level of consciousness	0 1 2 3 4
2. Weight Loss – unintentional >5%	0 1 2 3 4	25. Acute psychosis, delirium, confusion	0 1 2 3 4
3. Lymphadenopathy/Splenomegaly	0 1 2 3 4	26. Seizures	0 1 2 3 4
4. Fatigue/Malaise/Lethargy	0 1 2 3 4	27. Stroke or stroke syndrome	0 1 2 3 4
5. Anorexia/nausea/vomiting	0 1 2 3 4	28. Aseptic Meningitis	0 1 2 3 4
Mucocutaneous - MUST BE SLE RELATED		29. Mononeuritis multiplex	0 1 2 3 4
6. Maculopapular eruption – severe, active, (bullous)	0 1 2 3 4	30. Ascending or transverse myelitis	0 1 2 3 4
7. Maculopapular eruption – mild	0 1 2 3 4	31. Peripheral or cranial neuropathy	0 1 2 3 4
8. Active discoid lesions – generalized / extensive	0 1 2 3 4	32. Disc swelling/cytoid bodies	0 1 2 3 4
9. Active discoid lesions – localized including lupus profundus	0 1 2 3 4	33. Chorea	0 1 2 3 4
10. Alopecia (severe, active)	0 1 2 3 4	34. Cerebellar ataxia	0 1 2 3 4
11. Alopecia (mild)	0 1 2 3 4	35. Headache severe, unremitting	0 1 2 3 4
12. Panniculitis (severe)	0 1 2 3 4	36. Organic depressive illness	0 1 2 3 4
13. Angioedema	0 1 2 3 4	37. Organic brain syndrome including	0 1 2 3 4
14. Extensive mucosal ulceration	0 1 2 3 4	Pseudotumor cerebri	0 1 2 3 4
15. Small mucosal ulcers	0 1 2 3 4	38. Episodic migranous headaches	0 1 2 3 4
16. Malar erythema	0 1 2 3 4	**Musculoskeletal - MUST BE SLE RELATED**	
17. Subcutaneous nodules	0 1 2 3 4	39. Definite myositis (Bohan & Peter)	0 1 2 3 4
18. Perniotic Skin Lesions	0 1 2 3 4	40. Severe Polyarthritis with loss of function	0 1 2 3 4
19. Periungal erythema	0 1 2 3 4	41. Arthritis	0 1 2 3 4
20. Swollen fingers	☐Yes ☐No	42. Tendonitis	0 1 2 3 4
21. Sclerodactyly	☐Yes ☐No	43. Mild chronic myositis	0 1 2 3 4
		44. Athralgia	0 1 2 3 4
22. Calcinosis	☐Yes ☐No	45. Myalgia	0 1 2 3 4
23. Telangiectasia	☐Yes ☐No	46. Tendon contractures and fixed deformity	☐Yes ☐No
		47. Aseptic necrosis	☐Yes ☐No

Cardiovascular & Respiratory - MUST BE SLE RELATED		**Renal - MUST BE SLE RELATED**		(√) if SLE Related
48. Pleuropericardial pain	0 1 2 3 4	68. Systolic Blood Pressure (Enter value)	_____mm-Hg	☐
49. Dyspnea	0 1 2 3 4	69. Diastolic Blood Pressure (Enter value)	_____mm-Hg	☐
50. Cardiac Failure	0 1 2 3 4	70. Accelerated Hypertension	☐Yes ☐No	☐
51. Friction Rub	0 1 2 3 4	71. Urine dipstick (Enter value) (- = 0) (+ = 1) (++ = 2) (+++ = 3)		☐
52. Effusion (pericardial or pleural)	0 1 2 3 4	72. Urinary protein (Record a or b) a. 24 hr urinary protein b. Urine protein-creatine ratio	a _____ g b _____mm/mmol	☐
53. Mild or intermittent chest pain	0 1 2 3 4	73. Proteinuria (Record a or b) a. Newly documented proteinuria of > 1g/24 hours b. Newly documented protein-creatine ratio of >100mg/mmol	a. ☐Yes ☐No b. ☐Yes ☐No	
54. Progressive CXR changes – lung fields *If Not Done,√ NO on EDC BILAG	☐Yes OR Circle: No / Not Done	74. Nephrotic Syndrome	☐Yes ☐No	☐
55. Progressive CXR changes – heart size *If Not Done,√ NO on EDC BILAG	☐Yes OR Circle No / Not Done	75. Creatinine (serum) (Enter value)	_____mg/dl	☐
56. ECG evidence of pericarditis or Myocarditis *If Not Done,√ NO on EDC BILAG	☐Yes OR Circle No / Not Done	76. Creatinine clearance/GFR (Enter value)	_____ml/min	☐
57. Cardiac dysrhythmias including tachycardia >100 in the absence of fever *If Not Done,√ NO on EDC BILAG	☐Yes OR Circle No / Not Done	77. Active urinary sediment	☐Yes ☐No	☐
58. Pulmonary function fall by 20% *If Not Done,√ NO on EDC BILAG	☐Yes OR Circle No / Not Done	78. Histological evidence of active Nephritis - within 3 months	☐Yes ☐No	
59. Cytohistological evidence of inflammatory lung disease *If Not Done,√ NO on EDC BILAG	☐Yes OR Circle No / Not Done	86. Evidence of circulating anticoagulant	☐Yes ☐No	
Vascular - MUST BE SLE RELATED		**Hematology - MUST BE SLE RELATED**		
60. Major cutaneous vasculitis incl. ulcers	0 1 2 3 4	79. Hemoglobin (g/dl) (Enter value)	_____g/dl	☐
61. Major abdominal crisis due to vasculitis	0 1 2 3 4	80. Total white cell count (x 10^9/L) (Enter value)	_____x 10^9/L	☐
62. Recurrent thromboembolism excluding strokes	0 1 2 3 4	81. Neutrophils (x 10^9/L) (Enter value)	_____ x 10^9/L	☐
63. Raynaud's	0 1 2 3 4	82. Lymphocytes (x 10^9/L) (Enter value)	_____ x 10^9/L	☐
64. Livido reticularis	0 1 2 3 4	83. Platelets (x 10^9/L) (Enter value)	_____ x 10^9/L	☐
65. Superficial phlebitis	0 1 2 3 4	84. Evidence of active hemolysis	☐Yes ☐No	☐
66. Minor cutaneous vasculitis (nailfold vascultitis, digital vasculitis)	0 1 2 3 4	85. Coombs test positive	☐Yes ☐No	
67. Thromboembolism (excl. stroke) (first episode)	☐Yes ☐No	86. Evidence of circulating anticoagulant	☐Yes ☐No	

Appendix 2

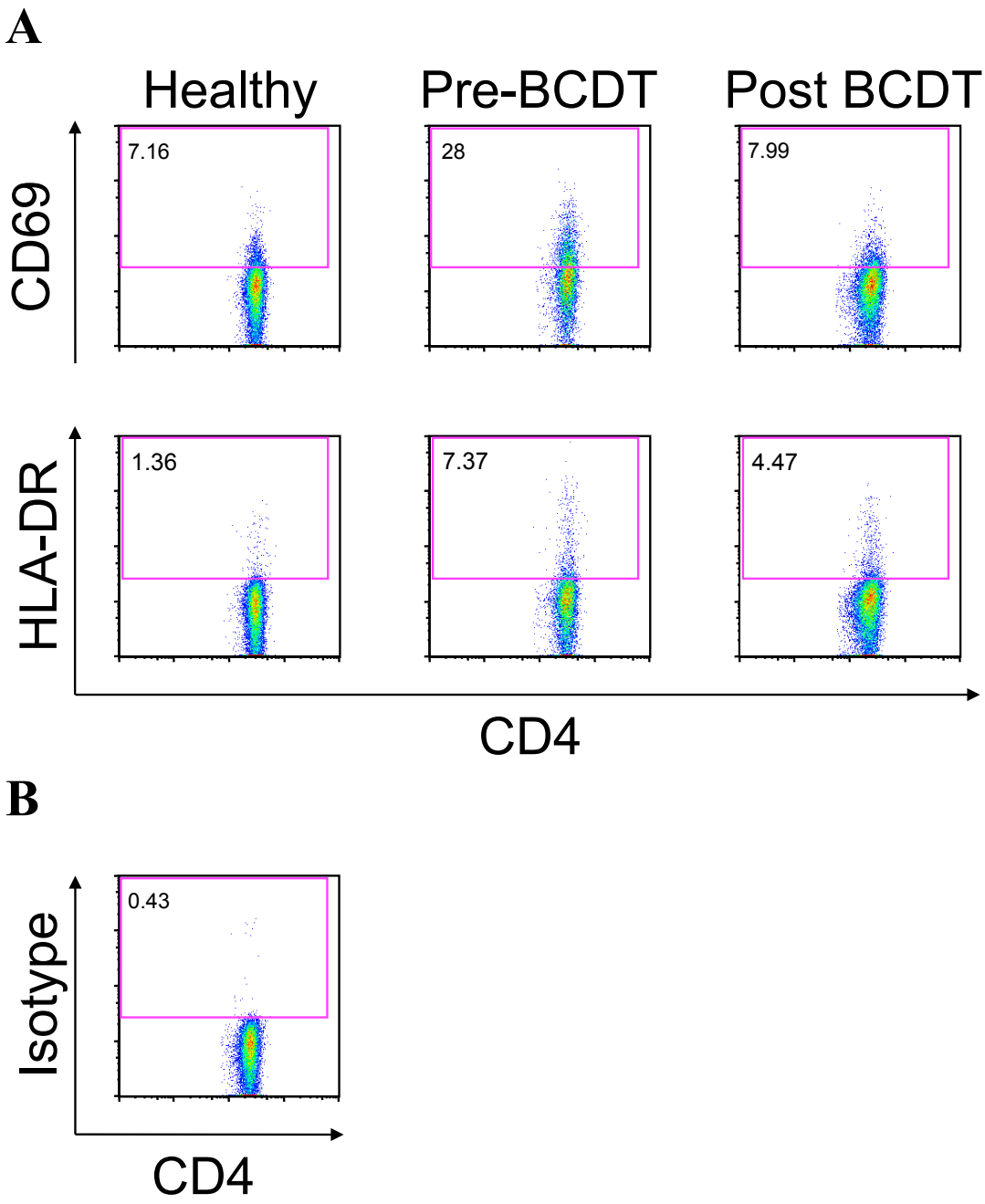


Figure 7.1.1 – Expression of CD69 and HLA-DR by $CD4^+$ T cells

(**A**) Representative flow cytometry dot plots illustrating the expression of CD69 and HLA-DR by $CD4^+$ T cells in a healthy individual and patient 29 before and after BCDT. The lymphocyte population was gated as described in chapter 3.2.8 (Methods). (**B**) Samples stained with the relative isotype control monoclonal antibody were analyzed concurrently to determine the positive gate.

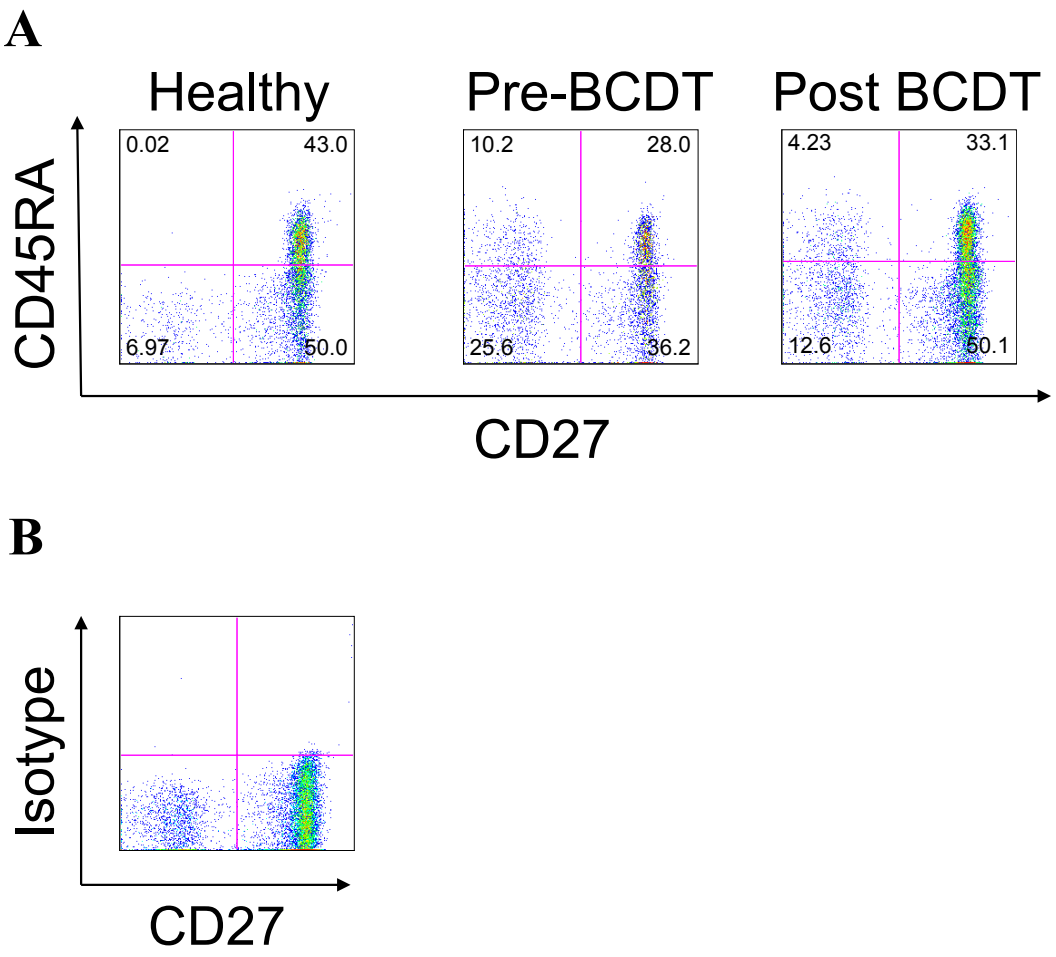


Figure 7.1.2 – Expression of CD45RA and CD27 by $CD4^+$ T cells

(**A**) Representative flow cytometry dot plots illustrating $CD4^+$ T cell memory subsets defined by CD45RA and CD27 expression in a healthy individual and patient 29 before and after BCDT. The lymphocyte population was gated as described in chapter 3.2.8 (Methods). (**B**) Samples stained with CD27 and the relative isotype control monoclonal antibody were analyzed concurrently to determine the positive gate.